

Second Edition

3D Bioprinting and Nanotechnology in Tissue Engineering and Regenerative Medicine

Edited by
Lijie Grace Zhang
John P. Fisher
Kam W. Leong



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Lijie Grace Zhang

*Department of Mechanical and Aerospace Engineering,
The George Washington University, Washington, DC, United States*

John P. Fisher

*Fischell Department of Bioengineering, University of Maryland,
College Park, MD, United States*

Kam W. Leong

*Department of Biomedical Engineering, Columbia University,
New York, NY, United States*



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List of contributors

Anthony Atala

Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, Winston-Salem, NC, United States

Amir Azhari

Department of Mechanical and Mechatronics Engineering, University of Waterloo, Waterloo, ON, Canada

Ahmad Basalah

Department of Mechanical and Mechatronics Engineering, University of Waterloo, Waterloo, ON, Canada

David Berry

Department of NanoEngineering, University of California, San Diego, CA, United States

Diana Bonilla

Division of Engineering of Medicine, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Cambridge, MA, United States

Nathan J. Castro

Department of Mechanical and Aerospace Engineering, The George Washington University, Washington, DC, United States

Robert C. Chang

Department of Mechanical Engineering, Stevens Institute of Technology, Hoboken, NJ, United States

Shaochen Chen

Department of NanoEngineering, University of California, San Diego, CA, United States

Boris Chichkov

Institute of Quantum Optics, Leibniz University Hannover, Hannover, Germany; Lower Saxony Centre for Biomedical Engineering, Implant Research and Development, Hannover, Germany

Douglas B. Chrisey

Department of Physics and Engineering Physics, Tulane University, New Orleans, LA, United States

Joshua Copus

Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, Winston-Salem, NC, United States

David T. Corr

Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY, United States

David Dean

Department of Materials Science and Engineering; Department of Plastic and Reconstructive Surgery, The Ohio State University, Columbus, OH, United States

Heng Deng

Department of Mechanical and Aerospace Engineering, University of Missouri, Columbia, MO, United States

Andrew D. Dias

R&D, FUJIFILM Cellular Dynamics, Inc, Madison, WI, United States

Erik W. Dorthé

Shiley Center for Orthopaedic Research and Education at Scripps Clinic, La Jolla, CA, United States

Darryl D. D'Lima

Shiley Center for Orthopaedic Research and Education at Scripps Clinic, La Jolla, CA, United States

Elizabeth D. Ferrill

Finnegan, Henderson, Farabow, Garrett & Dunner, LLP, Washington, DC, United States

John P. Fisher

Fischell Department of Bioengineering, University of Maryland, College Park, MD, United States; Center for Engineering Complex Tissues, University of Maryland, College Park, MD, United States

Warren Grayson

Department of Biomedical Engineering, School of Medicine, Johns Hopkins University, Baltimore, MD, United States; Translational Tissue Engineering Center, School of Medicine, Johns Hopkins University, Baltimore, MD, United States; Department of Materials Science & Engineering, Whiting School of Engineering, Johns Hopkins University, Baltimore, MD, United States; Department of Chemical & Biomolecular Engineering, Whiting School of Engineering, Johns Hopkins University, Baltimore, MD, United States; Institute for NanoBioTechnology (INBT), Johns Hopkins University School of Engineering, Baltimore, MD, United States

Bagrat Grigoryan

Department of Bioengineering, Rice University, Houston, TX, United States

Shawn P. Grogan

Shiley Center for Orthopaedic Research and Education at Scripps Clinic, La Jolla, CA, United States

Sung Yun Hann

Department of Mechanical and Aerospace Engineering, The George Washington University, Washington, DC, United States

Benjamin Holmes

Department of Mechanical and Aerospace Engineering, The George Washington University, Washington, DC, United States

Yong Huang

Department of Mechanical & Aerospace Engineering, University of Florida, Gainesville, FL, United States

Rita Kandel

Mount Sinai Hospital, University of Toronto, Toronto, ON, Canada

Megan Kimicata

Department of Materials Science and Engineering, University of Maryland, College Park, MD, United States; Center for Engineering Complex Tissues, University of Maryland, College Park, MD, United States

David M. Kingsley

Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY, United States

Lothar Koch

Institute of Quantum Optics, Leibniz University Hannover, Hannover, Germany; Lower Saxony Centre for Biomedical Engineering, Implant Research and Development, Hannover, Germany

Joel Kopcow

Shiley Center for Orthopaedic Research and Education at Scripps Clinic, La Jolla, CA, United States

Michael Larsen

Plastic Surgeon & Reconstructive Hand Surgeon, Arizona Center for Hand to Shoulder Surgery, Phoenix, AZ, United States

Jia Min Lee

School of Mechanical and Aerospace Engineering, Nanyang Technological University, Singapore

Sang Jin Lee

Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, Winston-Salem, NC, United States

Jian Lin

Department of Mechanical and Aerospace Engineering, University of Missouri, Columbia, MO, United States

Wai Cheung Ma

School of Mechanical and Aerospace Engineering, Nanyang Technological University, Singapore; Singapore Centre for 3D Printing (SC3DP), School of Mechanical and Aerospace Engineering, Nanyang Technological University, Singapore

Xuanyi Ma

Department of NanoEngineering, University of California, San Diego, CA, United States

Sushila Maharjan

Division of Engineering of Medicine, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Cambridge, MA, United States

Stefanie Michael

Department of Plastic, Hand- and Reconstructive Surgery, Hannover Medical School, Hannover, Germany

Jordan S. Miller

Department of Bioengineering, Rice University, Houston, TX, United States

Kathleen Miller

Department of NanoEngineering, University of California, San Diego, CA, United States

Michael Miller

Plastic and Reconstructive Surgeon, Banner MD Anderson Cancer Center, Gilbert, AZ, United States

Ruchi Mishra

Former Faculty at National Institute of Technology, Raipur, Chhattisgarh, India

Christopher O'Brien

Department of Mechanical and Aerospace Engineering, The George Washington University, Washington, DC, United States

Theresa B. Phamduy

Department of Biomedical Engineering, Tulane University, New Orleans, LA, United States

Jesse K. Placone

Department of Physics and Engineering, West Chester University, West Chester, PA, United States; Fischell Department of Bioengineering, University of Maryland, College Park, MD, United States

Kai Rajan

Finnegan, Henderson, Farabow, Garrett & Dunner, LLP, Washington, DC, United States

Kerstin Reimers

Department of Plastic, Hand- and Reconstructive Surgery, Hannover Medical School, Hannover, Germany

Cassandra L. Roberge

Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY, United States

Jayant Saksena

Department of Physics and Engineering Physics, Tulane University, New Orleans, LA, United States

Naboneeta Sarkar

Department of Biomedical Engineering, School of Medicine, Johns Hopkins University, Baltimore, MD, United States; Translational Tissue Engineering Center, School of Medicine, Johns Hopkins University, Baltimore, MD, United States

Yin-Lin Shen

Department of Mechanical and Aerospace Engineering, The George Washington University, Washington, DC, United States

Rohan Shirwaiker

Department of Industrial and Systems Engineering, North Carolina State University, Raleigh, NC, United States

S.C. Sklare

Department of Physics and Engineering Physics, Tulane University, New Orleans, LA, United States

Binil Starly

Department of Industrial and Systems Engineering, North Carolina State University, Raleigh, NC, United States

Sarah Strauß

Department of Plastic, Hand- and Reconstructive Surgery, Hannover Medical School, Hannover, Germany

Min Tang

Department of NanoEngineering, University of California, San Diego, CA, United States

Filippos Tournomousis

Department of Mechanical Engineering, Stevens Institute of Technology, Hoboken, NJ, United States

Ehsan Toyserkani

Department of Mechanical and Mechatronics Engineering, University of Waterloo, Waterloo, ON, Canada

Mihaela Vlasea

Department of Mechanical and Mechatronics Engineering, University of Waterloo, Waterloo, ON, Canada

Peter M. Vogt

Department of Plastic, Hand- and Reconstructive Surgery, Hannover Medical School, Hannover, Germany

Wai Yee Yeong

School of Mechanical and Aerospace Engineering, Nanyang Technological University, Singapore; Singapore Centre for 3D Printing (SC3DP), School of Mechanical and Aerospace Engineering, Nanyang Technological University, Singapore

Lijie Grace Zhang

Department of Mechanical and Aerospace Engineering, The George Washington University, Washington, DC, United States

Yu Shrike Zhang

Division of Engineering of Medicine, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Cambridge, MA, United States

Yuxiao Zhou

Department of Biomedical Engineering, School of Medicine, Johns Hopkins University, Baltimore, MD, United States; Translational Tissue Engineering Center, School of Medicine, Johns Hopkins University, Baltimore, MD, United States

Wei Zhu

Department of Mechanical and Aerospace Engineering, The George Washington University, Washington, DC, United States

Preface

This second edition of *3D Bioprinting and Nanotechnology in Tissue Engineering and Regenerative Medicine* aims to provide an overview of the exciting emergent technologies—3D/4D bioprinting and nanotechnology—for tissue and organ regeneration applications. It includes two main sections: (1) a thorough overview of current advancements in 3D/4D bioprinting and nanomaterials and (2) 3D/4D bioprinting of complex tissues and the regulatory implications of associated intellectual property. The discerning feature of this text compared with existing titles lies in the breadth of coverage of recent developments of these advanced technologies separately by leading researchers in the field. In addition, the most beneficial feature to current and future clinicians, students, researchers, and technologists is the depth and emphasis on combinatorial approaches of 3D/4D bioprinting and nanotechnology in addressing complex tissue defects with implications on the fabrication of functional organs.

Historically, scaffold-based approaches have led to a better understanding of the effects of 3D microenvironments and geometric cues on cell fate and resultant tissue/organ formation. Traditional scaffold fabrication methods (i.e., electrospinning, gas foaming, and particle-leaching) have shown to be sufficient for simple, single-tissue regenerative applications but lack the sophistication and flexibility in design to fabricate more complex and biomimetic 3D structures suitable to support the creation of multicellular tissues. To address the biomimetic design, tissue engineers have begun to explore additive manufacturing, particularly 3D printing, as a viable method of fabricating tissue-engineered constructs. Innovative applications of existing 3D printing platforms, previously utilized commercially, as well as tissue-engineering-specific systems, have begun to be employed in earnest as tools to address several persistent limitations of traditional tissue construct fabrication methods. 3D bioprinting readily provides greater precision and control over the pre-designed internal architecture and composition of a scaffold when compared with the aforementioned traditional techniques. In addition, based on the computer-aided designed models reconstructed from noninvasive medical imaging data specific to individual patients' defects, 3D bioprinters can easily fabricate a custom-designed tissue construct in a layer-by-layer manner, which would allow the implant to perfectly integrate with the wound site and expedite tissue regeneration *in vivo*. Furthermore, as a new paradigm in additive manufacturing, 4D printing is an emerging, time-dependent manufacturing process to fabricate multifunctional smart structures with a dynamic capacity when exposed to an external stimulus. Compared with 3D bioprinting, the addition of a “time” dimension in bioprinting is very intriguing in the view that a dynamic 4D effect can better mimic natural tissue development to regulate cell behaviors. Through the commoditization of 3D printers and extension of these exciting technologies toward regenerative applications in concert with developing and evolving 3D/4D bioprinting-specific biomaterials, the potential for expedited regulatory oversight can lead to a standardization of biomimetic and smart implantable tissue scaffolding. The current (second) edition will provide an in-depth and up-to-date survey of 3D bioprinting and emerging 4D bioprinting for tissue and organ regeneration.

Complementing the architectural control afforded by 3D printing, nanotechnology offers local control at the site of cell–substrate interactions. Natural tissue, such as extracellular matrix, is full of nanoscale features in the form of nanofibers, nanopores, and nanoridges. These nanostructures play a key role in modulating the repair and regeneration of tissues. Through this book, the readers

will learn the most recent effort to exploit the dramatic effects of nanoscale features and novel nanomaterials on cell behavior and subsequent tissue formation. Nanomaterials with a feature size below 100 nm in at least one dimension can drastically alter the characteristics of a bulk material even at a low concentration in the form of a composite, ranging from mechanical to electrical properties. In addition to augmenting material properties, many nanomaterials may improve biological properties. The unique properties of these nanobiomaterials offer tissue engineers exciting opportunities to construct a biomimetic microenvironment for cellular studies and regenerative medicine applications.

In essence, tissue engineering thrives at the confluence of engineering and life sciences to solve important medical problems. Tissue engineers must effectively apply cutting-edge and multidisciplinary approaches in an integrated and novel manner. As these research areas evolve and are developed into proven technologies and eventually medical remedies, intellectual property considerations will apply and have to be addressed. For this reason, a chapter has been included to highlight the role of intellectual property for bioprinting and nanotechnology in tissue engineering. Together, these chapters form a unique and comprehensive book that we believe will be useful to not only understand recent breakthroughs and ongoing challenges in how 3D/4D printing and nanotechnology may impact cell–material interactions but also spur the cultivation of new strategies to exploit these technologies for the translation and commercialization of tissue engineering.

Lijie Grace Zhang
John P. Fisher
Kam W. Leong

Nanotechnology: A Toolkit for Cell Behavior

Christopher O'Brien, Sung Yun Hann, Benjamin Holmes and Lijie Grace Zhang

Department of Mechanical and Aerospace Engineering, The George Washington University, Washington, DC, United States

1.1 INTRODUCTION

Scientists and researchers have been fascinated with the details of life at small scales ever since Robert Hooke saw the evidence of small structures in cork that he coined cells. This spurred the creation of the compound microscope and the quest of the late 1600s to discover how life operates beneath our very own eyes. That quest has continued, even to this day as scientists look for smaller and smaller constituents that contribute to life as we know it; from proteins to functional groups, everything has an important role. The collective scientific gaze looked for finer and finer components to life, and for a short while now has focused on the prevalence of the nanoworld.

One hundred to one thousand times smaller than Hooke's observed cork cells, researchers have determined that materials and features of <100 nm in at least one dimension can have profound effects on the behavior of cells and further tissue and organ regeneration (Zhang & Webster, 2009). When examining nature, using nanotechnology for tissue regeneration becomes obvious. In fact, human cells create and continually interact directly with their natural nanostructured environment, called extracellular matrix (ECM). This momentous discovery spurred many researchers to attempt to more effectively mimic natural biology by creating novel nanobiomaterials and designing nanocomposite scaffolds for improved tissue and organ regenerations (Biggs, Richards, Gadegaard, Wilkinson, & Dalby, 2007; Chopra, Mummery, Derby, & Gough, 2012; Cui, Nowicki, Fisher, & Zhang, 2017; Cui et al., 2018; Jang, Namgung, Hong, & Nam, 2010). Decreasing material size to the nanoscale dramatically increases surface roughness and the surface area to volume ratio of materials, and may lead to a higher surface reactivity and many superior physiochemical properties (*i.e.*, mechanical, electrical, optical, catalytic, and magnetic properties) (Zhang & Webster, 2009). The excellent properties of nanobiomaterials make them hold great potential for a wide range of biomedical applications, particularly advanced tissue/organ regeneration.

With the exponential growth in population and the similarly rapid increase in lifespan worldwide, there is an enormous market for various tissue and organ transplantations and engraftments. Current treatment options for damaged tissues and organs are nonideal, and often involve severe tissue/organ shortages, painful surgeries, and long recovery times without offering a complete restoration of the tissue's and organ's function. Simultaneously, in recent years, many health professionals are advocating for a more active lifestyle with increased exercise and thus an increased risk

of injury. These factors, and many others, put a strain on existing treatment methods and hallmark their many weaknesses. For most tissue damage caused by diseases or injuries, many current treatment methods lack the ability to restore the affected area to a level of functionality equivalent to healthy native tissue. They instead provide a stop-gap, or temporary solution that either slows the progress of further degeneration, or requires a sacrifice of other healthy tissue (autografts). It is the hope of many researchers and doctors alike that through increasing understanding of how cells and tissue interact on the nanoscale and creating biomimetic nanostructured tissue constructs to better emulate natural designs, solutions can be discovered that more effectively treat diseases and injuries.

In the following sections, we will focus on the current state of nanotechnology for a series of tissue and organ regeneration. In addition, we will put special emphasis on integrating cutting-edge 3D nano/microfabrication techniques with nanobiomaterials for complex tissue and organ regeneration applications. These nanobiomaterial constituents can be made of nearly any material imaginable, including carbon nanomaterials, self-assembly nanomaterials, natural or synthetic polymers, ceramics, drug-containing spheres, or metal particles. Researchers strive to combine the appropriate nanobiomaterials, cells, and growth factors to create the ideal biomimetic tissue-engineered construct that could surmount traditional methods of injury mitigation.

1.2 NANOBOMATERIALS FOR TISSUE REGENERATION

1.2.1 CARBON NANOBOMATERIALS

1.2.1.1 *Carbon Nanotubes*

Carbon and carbon derivatives are some of the most versatile nanomaterials that tissue engineers have in their arsenal (Harrison & Atala, 2007; Peng et al., 2020; Tran, Zhang, & Webster, 2009; Zhang, Ercan, & Webster, 2009). In addition to constituting 18% of the average human body by mass (Frieden, 1972), carbon is a highly flexible element that can assume many nanometer-sized structures. One of the most well-explored carbon nanomaterials is carbon nanotubes (CNTs, Figure 1.1). CNTs have several different types but those used in the tissue engineering field are primarily multiwalled carbon nanotubes (MWCNTs) or single-wall carbon nanotubes (SWCNTs). They are one of the strongest materials known (Terrones, 2004; Yu et al., 2000) and can exhibit both semiconducting (Jung et al., 2013) and conducting (Lan & Li, 2013) properties, making them interesting media for stimulating tissue regeneration.

One of the most prominent features of CNTs is their ability to significantly influence the electrical conductivity of scaffolds. This trait is of particular interest to groups studying tissues that rely heavily on signaling to perform functions, like cardiac tissue. In one example, gelatin methacrylate (GelMA) hydrogel scaffolds modified with incorporated CNTs expressed improved cell behavior when seeded with rat cardiomyocytes. The tissue exhibited increased synchronous beating rate, and a significantly lower threshold for excitation when compared to control samples without incorporated CNTs (Shin et al., 2013). Furthermore, CNTs also tend to increase cardiomyocyte proliferation and maturation *in vitro* (Martinelli et al., 2012, 2013; Shin et al., 2013). Although CNTs are used for several other fields within tissue engineering, they appear to selectively steer mesenchymal

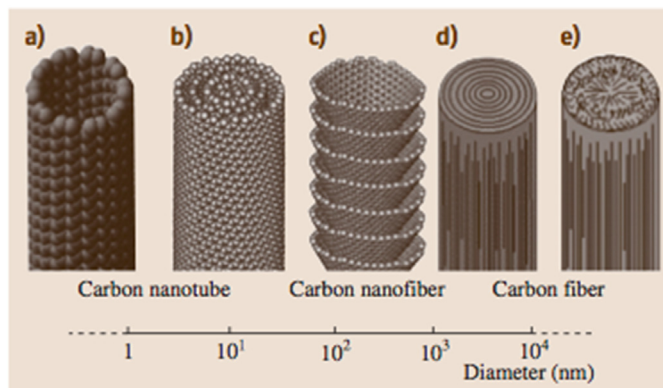


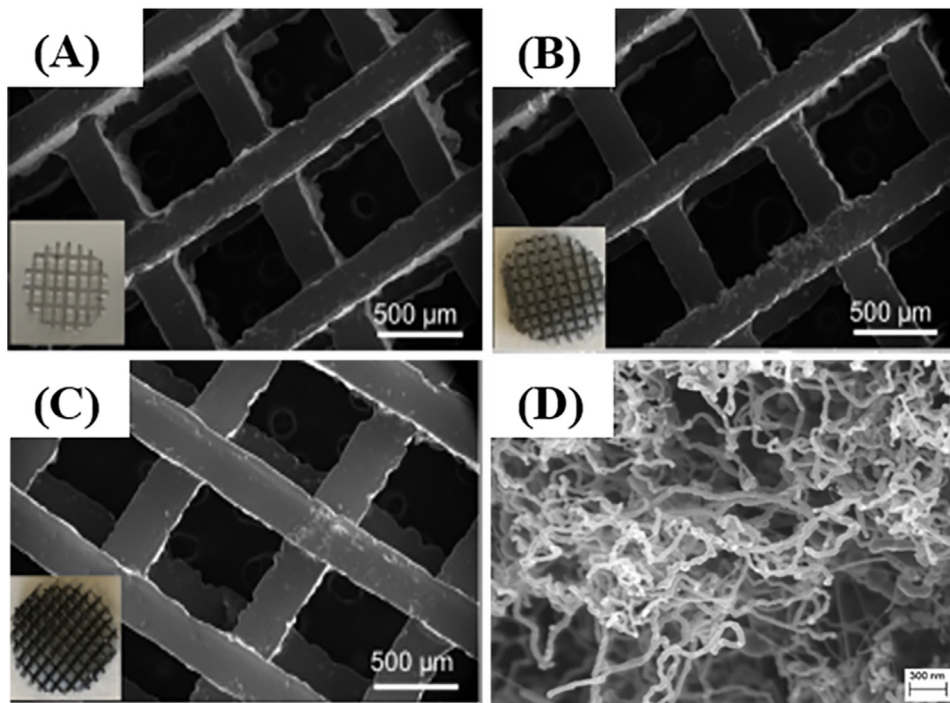
FIGURE 1.1

Comparison of carbon nanotubes and carbon nanofibers showcasing their morphological differences, and the relative difference in diameter.

Image is adapted with permission from Kim et al. (2013).

stem cells (MSCs) toward a cardiac lineage when introduced into cell culture media and exposed to electrical stimulation (Mooney et al., 2012).

Many researchers have also drawn upon the high electrical conductivity exhibited by CNTs to create conductive scaffolds for neural tissue regeneration (Gacem et al., 2013; Lee et al., 2018). Improved peripheral nervous system (Serrano et al., 2014), central nervous system (CNS) (Kim et al., 2014) regeneration, and stem cell performance (Serrano et al., 2014) have been observed when utilizing CNTs. In particular, an MWCNT embedded hydrogel-based scaffold was fabricated via 3D printing and coupled with regulated electrical pulse stimulation while a scaffold with no MWCNT content served as a control group (Figure 1.2A–C). As a result, improved differentiation and growth of neural stem cells (NSCs) as well as promoted neurite length were observed after 8 days of culture (Lee et al., 2018). In addition, a 3D porous scaffold was fabricated from chondroitin sulfate, a biomaterial constituent of native nervous tissue, and MWCNTs via a freeze-drying method, coated with polylysine, and cultured with rat embryonic neural progenitor cells. After 20 days of culture, a viable cell population of more neurons than glial cells was observed, contrasting the two dimensional (2D), CNT-less controls (Serrano et al., 2014). In another study, MWCNTs were combined with collagen to create a collagen-CNT nanomaterial scaffold that accelerated and directed differentiation of human decidua parietalis stem cells into a neural lineage (Sridharan, Kim, Strakova, & Wang, 2013). Another approach to engineer a double-layered nerve guidance conduit using MWCNT with the incorporation of chitosan grafted polymer and electrospinning (Shrestha et al., 2018). The nanocomposite scaffold elucidated a previously unknown differentiation pathway of parietalis cells, unique to this scaffold. In addition, CNTs can also be used to reveal important mechanisms of neuronal activity. In one study, MWCNTs with a small number of walls, dubbed “few-walled CNTs,” were used as a substrate to examine the chloride shift; a hallmark trait of neuronal disorder and injury (Liedtke et al., 2013). Primary CNS neurons were found to have a highly accelerated chloride shift and very high potassium chloride cotransporter 2 expression. The

**FIGURE 1.2**

Scanning electron microscopy (SEM) images of hydrogel scaffolds with different concentrations of embedded MWCNTs, (A) 0.02%, (B) 0.05%, and (C) 0.1%. (D) SEM image of hydrogen purified MWCNTs.

(A–C) Images are adapted with permission from (Lee et al. 2018). (D) Image is adapted with permission from (Holmes et al. 2013).

CNTs on the substrate promoted this expression through interaction with voltage-gated sodium channels. In this manner, few-walled CNTs can be employed to reduce chloride concentration and exchange between neurons.

CNTs continue to have great utility across many other tissue types. At first glance, the musculoskeletal system is very different from the cardiac and neuronal systems previously discussed; however, they are alike in that carbon nanotubes can provide important augmentations to biomaterial scaffolds to increase the efficacy of the constructs. In bone tissue engineering, the main concern of researchers attempting to create an ideal tissue-engineered scaffold is modulating the mechanical properties of biomaterials to be similar to that of natural tissue. They seek to not only strengthen scaffolds but also activate the mechanotransductive pathway that induces osteogenesis in human bone marrow MSCs (Engler, Sen, Sweeney, & Discher, 2006). By incorporating MWCNTs, researchers were able to not only increase the mechanical strength of poly(caprolactone) (PCL) scaffolds but also increase adhesion, proliferation, and differentiation of rat MSCs (Pan, Pei, He, Wan, & Wang, 2012). In addition, our laboratory created a new 3D nanocomposite scaffold based on magnetically treated SWCNTs, nanocrystalline hydroxyapatite (nHA), and chitosan hydrogel for

improved bone regeneration (Im, Li, Wang, Zhang, & Keidar, 2012). Human fetal osteoblasts adhered and proliferated more vigorously on nanoscaffolds with magnetically treated SWCNTs over nonmagnetically treated SWCNTs. Notably, the spreading morphology on the magnetically treated SWCNT augmented scaffolds showed extended filopodia, indicative of strong cell attachment. This effect was further explored by another study in our laboratory. A nanocomposite coating consisting of magnetically treated SWCNTs and nHA was created, and deposited onto titanium for analysis. Samples coated with SWCNTs exhibited increased MSC and osteoblast adhesion and proliferation when compared with uncoated controls, and samples treated with nonmagnetically treated SWCNTs (Wang, Castro, Li, Keidar, & Zhang, 2012). These papers also highlight the possible synergistic effect present when combining multiple nanomaterials within a single orthopedic implant. Moreover, Abarrategi et al. fabricated a scaffold using the freeze-drying method to create fibrous scaffolds consisting of up to 89% MWCNTs. Scaffolds performed well when seeded with myoblastic mouse cell C2C12 (with osteogenic potential) *in vitro* and supported favorable cellular adhesion and proliferation results. The nanoscaffolds were then implanted in a mouse subcutaneous muscular pocket defect, and showcased quick degradation and the beginnings of collagen formation at the interface of native tissue and the scaffold (Abarrategi et al., 2008).

Carbon nanotubes can also be leveraged to bolster very weak materials and render them usable for tissue engineering applications that otherwise would be less than ideal. Electrospinning (to be described in Section 3.1), a common nanoscaffold fabrication technique, can be used to generate tissue-engineered cartilage constructs (Holmes, Castro, Zhang, & Zussman, 2012; Karbasi & Alizadeh, 2017). However, the Young's modulus of the resulting scaffolds is often much lower than autologous tissue. To combat this, Holmes et al. used H₂ purified MWCNTs (Figure 1.2D) to create an electrospun nanocomposite scaffold with Young's modulus more similar to cartilage and, with the addition of a polylysine coating, improved chondrogenic differentiation of MSCs significantly when compared to controls (Holmes, Castro, Li, Keidar, & Zhang, 2013). Karbasi and Alizadeh enhanced mechanical properties of the fabricated cartilage tissues by the inclusion of poly (3-hydroxybutyrate)-chitosan into MWCNTs for the electrospinning process (Karbasi & Alizadeh, 2017).

1.2.1.2 Carbon Nanofibers

Similar to CNTs, carbon nanofibers consist of graphene sheets rolled into 3D structures with a cone or cylindrical shaped morphology. A carbon nanofiber can be defined as “a sp²-based linear filament with a diameter of 100 nm that is characterized by flexibility and their aspect ratio (above 100)” (Kim, Hayashi, Endo, & Dresselhaus, 2013) as seen in Figure 1.1. Carbon nanofibers are usually manufactured through vapor deposition with or without a metal catalyst (Endo, 1988), or less commonly, using a mechanical spinning process (Li, Kinloch, & Windle, 2004). Carbon nanofibers have been used throughout tissue engineering to improve mechanical properties and cellular activity, for multiple tissue regeneration applications.

For bone regeneration, Elias et al. reported that osteoblast proliferation and long-term functions (*i.e.*, synthesis of alkaline phosphatase and deposition of extracellular calcium up to 21 days) can be significantly improved on 60 nm diameter carbon nanofibers without a pyrolytic outer layer and 100 nm diameter carbon nanofibers with a pyrolytic outer layer compared with conventional larger carbon fibers (Elias, Price, & Webster, 2002). Since it is well known that surface properties (such as surface area, surface roughness, and the number of surface defects) of implant materials may

have important influences on cell functions (including adhesion, proliferation, differentiation, and mineralization) (Webster, Ergun, Doremus, Siegel, & Bizios, 2000; Zhang, Sirivisoot, Balasundaram, & Webster, 2008), enhanced long-term osteoblast functions on carbon nanofibers have been attributed to the special nanometer surface topography of carbon nanofibers, which mimic the dimension of inorganic crystalline hydroxyapatite and collagen in natural bone. For example, the study performed by Samadian et al. showed that the incorporation of carbon nanofibers as the basis for electrical stimulation of bone cells contributed to inducing promoted bone growth as well as osteogenic activity of the cells (Samadian et al., 2020).

Carbon nanofibers are also attractive in neural tissue engineering due to their excellent structural and electrical properties, and from a practical perspective are relatively inexpensive. One approach to utilize carbon nanofibers is to attach or grow them on a conductive polymer to create an array of fibers, and then seed cells onto the construct (Nguyen-Vu et al., 2007). This not only allows for improved electrical conductivity of the material but the neural cells also demonstrated good electrical contact with the nanofibers. Leveraging these observed advantages, carbon nanofibers were injected into stroke-damaged rat brain defects. The rats that were injected with NSCs in tandem with carbon nanofibers formed significantly less glial scar tissue when compared to positive and negative controls, indicating a more successful neural repair (Lee, Khang, Kim, & Webster, 2006).

1.2.1.3 Graphene

Graphene is the simplest nanomaterial form of carbon, and functions as a base unit for all other carbon nanomaterials. It consists of one monolayer of carbon atoms bonded in sp^2 hybridization orbitals. Sought after as a nanomaterial constituent of composite materials, graphene has a high elastic modulus, high electrical conductivity, and the potential to increase nanotexturization of surfaces. These characteristics apply strongly to many different kinds of tissue engineering, including but not limited to bone, cartilage, and nerve.

Because graphene makes up carbon nanotubes, it is possible to create graphene by “unzipping” carbon nanotubes, a fact that Akhavan et al. took advantage of. They used a silicon dioxide (SiO_2) matrix doped with titanium dioxide (TiO_2) nanoparticles as a photostimulation agent to utilize unzipped MWCNTs to form a graphene nanogrid atop a matrix. The researchers then tested the cellular response to this material using human NSCs cultured for 3 weeks. NSCs responded favorably to the growth surface, proliferated faster, and had a strong affinity toward neuronal differentiation lineages when grown on the graphene nanogrids. Additionally, the TiO_2 nanoparticles accelerated differentiation in the neuronal lineage when exposed to flash photostimulation (Akhavan & Ghaderi, 2013). The experiment showed that graphene can not only improve neuronal differentiation from NSCs but also work in concert with TiO_2 nanoparticles, strongly implying that it is possible to synergistically couple graphene with other nanomaterials for enhanced performance. Similarly, the use of graphene can be applied to a different cell line to obtain the same results. Jakus et al. have shown that a 3D-printed graphene composite can support MSC proliferation and neurogenic differentiation both *in vitro* and *in vivo* at least over 14 and 30 days, respectively (Jakus et al., 2015).

The high mechanical strength of graphene leads one to think of it as an ideal material for bone and cartilage tissue engineering, but it inherently lacks sufficient 3D structure for use as a bulk material in tissue engineering. One laboratory has developed a method to fabricate graphene foams, circumventing this typical problem. First, nickel foam was created and used as a substrate

to grow graphene via vapor deposition. The nickel was then dissolved away, leaving behind a 3D structure made entirely of multilayer graphene nanomaterials. Cell viability was observed over 14 days of culture, and osteogenic factors were measured after the 2-week period by fluorescent staining of osteopontin and osteocalcin (Crowder et al., 2013). The graphene foams not only supported MSC attachment but also spontaneously promoted osteogenesis without the addition of any osteogenic factors in the growth media (Crowder et al., 2013). In addition, one study performed in our laboratory revealed that the presence of graphene oxide in a biocompatible photocrosslinkable bioink for a hierarchical layer-by-layer 3D-printing process, consisting of GelMA and poly(ethylene glycol) diacrylate (PEG-DA) significantly contribute to chondrogenic differentiation of MSCs (Zhou et al., 2017). Likewise, this investigation shows that graphene in a form of graphene oxide blended with photocrosslinkable hydrogels could also be a great candidate to heal damaged cartilage. These are significant as they demonstrate the ability of graphene, in particular, to upregulate specific differentiation pathways without delivery of additional chemical cues. Our previous study also showed that electrospun PCL fibrous scaffolds with incorporated carbon nanotube/graphene could have a powerful effect on MSC fate as well. The scaffolds with carbon nanomaterial exhibited greatly increased MSC growth and glycosaminoglycan synthesis when compared to control, indicating great potential for cartilage repair (Holmes, Fang, Zarate, Keidar, & Zhang, 2016).

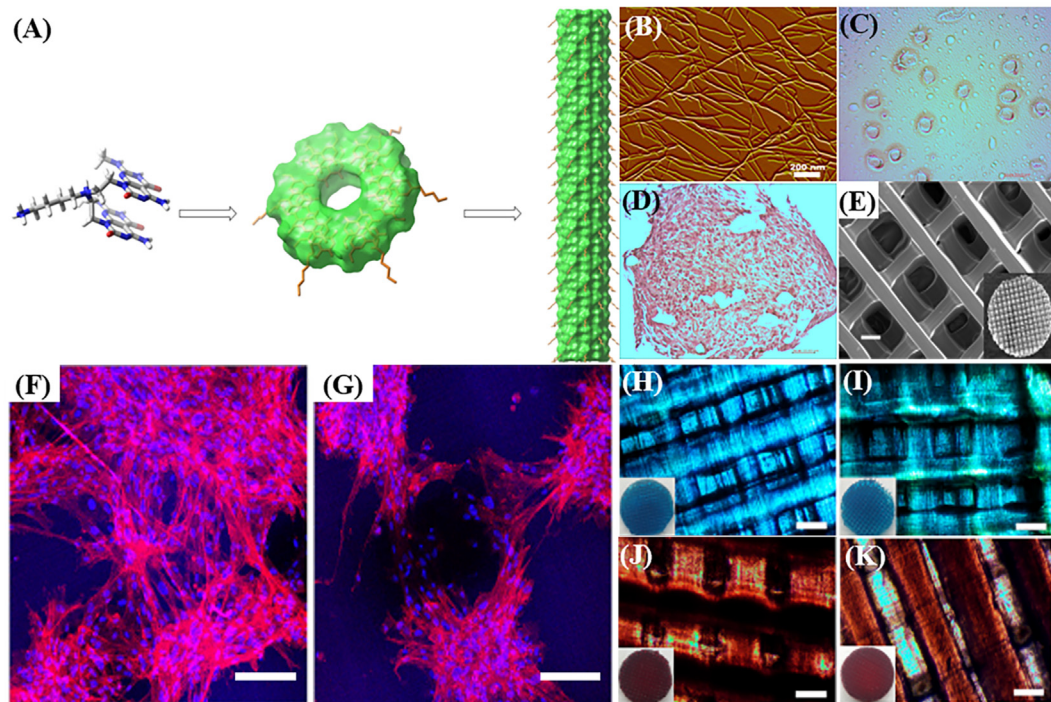
Much similar to graphene, black phosphorus (BP), which is a 2D-structured semiconducting material, recently has emerged as an alternative candidate in tissue engineering due to its exclusive properties, such as decent electrical conductivity and outstanding optical as well as mechanical properties (Choi et al., 2018). The applications of BP in tissue engineering can be found in the recently reported studies. Yang et al. integrated 2D BP nanosheets into 3D-printed scaffolds to provide a countermeasure for the photothermal treatment of osteosarcoma (Yang et al., 2018). Lui et al. also presented the synergetic effect of 3D-printed scaffolds coated with 2D BP and graphene oxide nanosheets for osteogenesis, which ultimately would result in the new bone formation (Liu et al., 2019).

1.2.2 SELF-ASSEMBLING NANOBIMATERIALS

Since natural tissues are constructed via a bottom-up self-assembly process, scientists are attempting to emulate natural ECM assembly via an emerging class of nanobiomaterials. These nanobiomaterials can self-assemble *in situ* from constituent groups into complex 3D structures on the nano- and microscale and hold great potential to facilitate the construction of complex, biomimetic tissue environments in a highly reproducible manner (Huebsch & Mooney, 2009). The sheer number of self-assembling nanobiomaterials is quite large and includes collagen, DNA, RNA, peptides, and many more (Zhang, 2003). Several self-assembling nanobiomaterials of particular interest will be discussed below.

1.2.2.1 Self-Assembling Nanotubes

Rosette nanotubes (RNTs, Figure 1.3A and B) are a class of biologically inspired supramolecular self-assembling nanomaterials. It consists of repeating units of DNA base pairs (Guanine^Cytosine) that assemble into rings (rosettes), that then stack axially to form hollow tubes with controllable 3–4 nm in diameter and lengths up to several microns. They are so versatile that they can be functionalized with amino acid, peptide, and small molecule side chains. Such side chain groups can be

**FIGURE 1.3**

(A) Schematic illustration of the self-assembly process of RNTs: Six twin DNA motifs are self-assembled into rosette-like supermacrocycles and then many of them stack up into stable helical nanotubes with a 3–4 nm diameter and several hundred nanometer long. Atomic force microscopy (AFM) images of (B) RNTs with aminobutane linker (TBL). Hematoxylin & Eosin staining of (C) controls; and (D) TBL scaffolds for cartilage regeneration at week 1. (E) An SEM image of the fabricated RNTK scaffold (0.05 mg/mL). Scale bar, 200 μ m. Confocal microscope images of ADSCs on the scaffolds with (F) and without RNTK (G) after 6 days. Scale bars, 200 μ m. Light microscope images of Alcian Blue stained ADSCs on the scaffolds with (H) and without RNTK (I) after 3 weeks of differentiation. Scale bars, 200 μ m. Light microscope images of Safranin-O stained ADSCs on the scaffolds with (J) and without RNTK (K) after 3 weeks of differentiation. Scale bars, 200 μ m.

(A, B) Images are adapted with permission from (Zhang et al. 2010). (C, D) Images are adapted with permission from (Childs et al. 2013). (E–K) Images are adapted with permission from (Zhou et al. 2020).

anything, such as cell-adhesive lysine, lysine-arginine-serine-arginine (KRSR), and arginine-glycine-aspartic acid-serine-lysine (RGDSK) for enhanced and directed osteoblast, chondrocyte, and MSC function, thus making them intriguing nanomaterials for many types of tissue regeneration (Fine, Zhang, Fenniri, & Webster, 2009; Sun, Zhang, Hemraz, Fenniri, & Webster, 2012; Zhang, Chen, Rodriguez, Fenniri, & Webster, 2008; Zhang, Hemraz, Fenniri, & Webster, 2010; Zhang, Rakotondradany, Myles, Fenniri, & Webster, 2009; Zhang, Ramsaywack, Fenniri, & Webster, 2008; Zhang, Rodriguez, et al., 2009). For instance, it has been demonstrated that RNTs can significantly enhance osteoblast growth and osteogenic differentiation when compared to controls (Sun et al., 2012). It was also observed that RNTs can directly nucleate and align nHA

particles along the long axis of the nanotubes similar to the self-assembled pattern of collagen and nHA in bone, suggesting that our nanotubes can serve as excellent templates for nHA nucleation (Zhang, Rodriguez, et al., 2009). In addition, by thoroughly modulating the RNTs peptide side chains, improved endothelial cell growth can be obtained on RNTs when compared to controls (Fine et al., 2009). In another study, MSC adhesion, proliferation, and 4 weeks of chondrogenic differentiation have been explored on twin-based RNTs embedded within poly-L-lactic acid scaffolds (Childs, Hemraz, Castro, Fenniri, & Zhang, 2013). The results demonstrated that these biomimetic twin-based nanotubes can significantly enhance MSC growth and chondrogenic differentiation, collagen, and protein synthesis (Figure 1.3C and D) when compared to controls without nanotubes. Recently, a three-layer gradient cartilage scaffold with lysine-functionalized RNTs was created using bioink formulations composed of varied ratios of GelMA and PEG-DA hydrogels for improving growth and chondrogenic differentiation of adipose-derived mesenchymal stem cells (ADSCs) (Figure 1.3E–K) (Zhou et al., 2020). After 3 weeks of differentiation, it was found that the collagen II, glycosaminoglycan, and total collagen content secreted by the differentiated ADSCs had increased by 59%, 71%, and 60%, respectively, on lysine-functionalized RNT (RNTK) gradient scaffolds with respect to the control group. Both the biomimetic nanostructure and high density of peptides with well-organized architecture contributed to the greatly enhanced stem cell functions *in vitro*.

1.2.2.2 Self-Assembling Nanofibers

Besides the aforementioned self-assembling nanotubes, natural peptides have demonstrated the ability to self-assemble into highly ordered, nanofibrous scaffolds in an aqueous solution. These peptides possess an ionic self-complementary structure derived from positive and negative side chains on one side of the β -sheet. In addition to this self-complementary system, the amphiphilic character, that is to say containing many hydrophobic and hydrophilic features, of the peptides enable hydrophilic interactions with water molecules. These unique features contribute to the formation of a hygroscopic nanofibrous hydrogel network, with the hydrophobic regions associated into a double sheet, resulting in the formation of the nanofibers (Hauser & Zhang, 2010). For instance, Hartgerink et al. reported that a self-assembly peptide-amphiphile with the cell-adhesive RGD (Arg-Gly-Asp) self-assembled into supramolecular nanofibers and aligned nHA on their long axis for bone application (Hartgerink, Beniash, & Stupp, 2001). Hosseinkhani et al. showed significantly enhanced osteogenic differentiation of stem cells in a 3D peptide-amphiphile scaffold compared to 2D static tissue culture (Hosseinkhani, Hosseinkhani, Tian, Kobayashi, & Tabata, 2006). In addition, Shah et al. designed peptide-amphiphile nanofibers which display a high density of transforming growth factor $\beta 1$ (TGF- $\beta 1$) binding sites for improved cartilage regeneration (Aida, Meijer, & Stupp, 2012; Shah et al., 2010). Similarly, Florine et al. cultured human and bovine MSCs in self-assembling peptide, and agarose hydrogels as control with TGF- $\beta 1$ and dexamethasone (Dex) for cartilage tissue regeneration (Florine et al., 2013). As a result, better-sulfated glycosaminoglycan (sGAG) retention and accumulation with higher proteoglycan synthesis were observed in the self-assembling peptide.

Simpler peptides can be leveraged as self-assembly nanomaterials for neural applications as well, such as isoleucine-lysine-valine-alanine-valine (IKVAV) (Silva et al., 2004) and RADA16 (Gelain, Bottai, Vescovi, & Zhang, 2006). Because the peptides used in self-assembled scaffolds are derived from biology, the resulting nanofibrous scaffolds are similar to natural ECM and

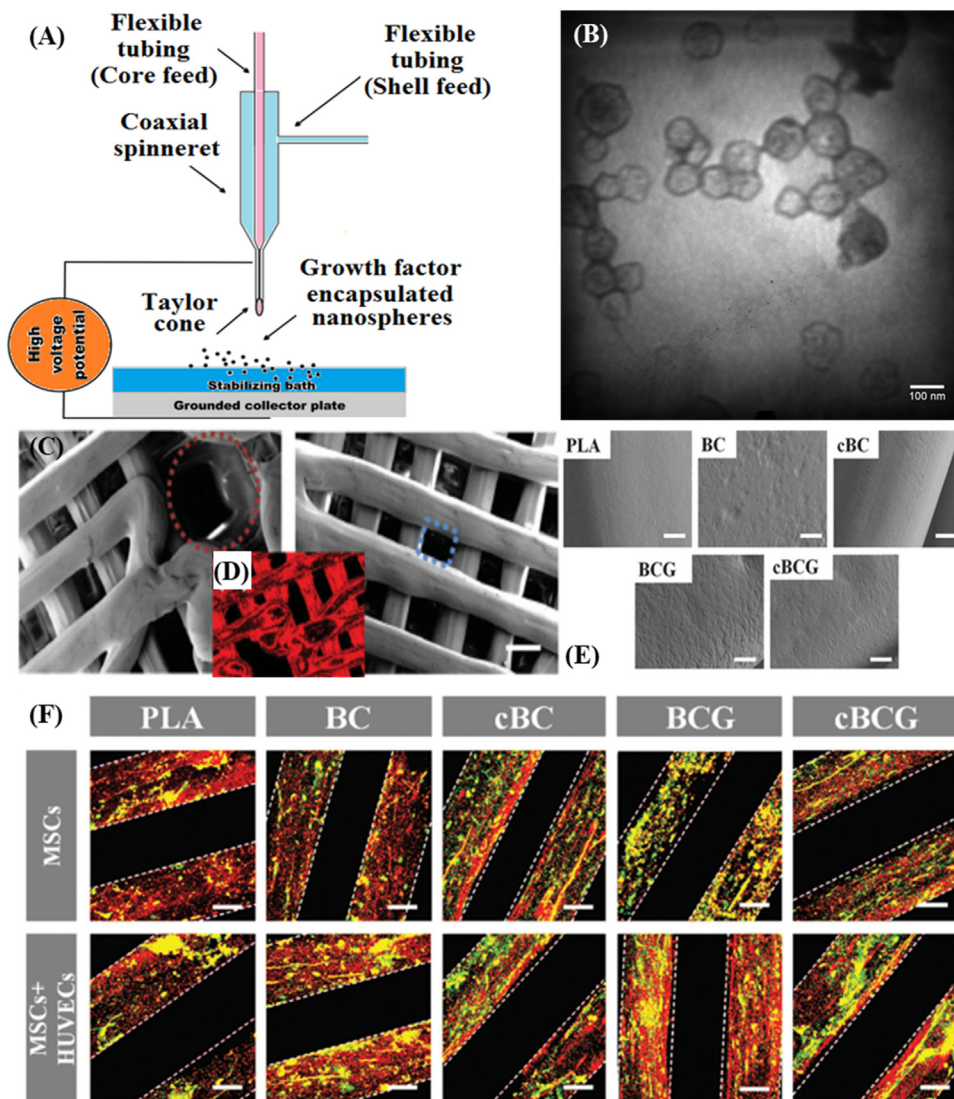
present excellent biomimetic properties. Peptide nanofibers have become the most widely investigated self-assembling nanobiomaterials for tissue engineering.

1.2.3 POLYMERIC AND CERAMIC NANOBOMATERIALS

1.2.3.1 Polymeric Nanobiomaterials

As the largest biomaterial group, polymers play an important role in complex tissue engineering. Polymeric nanobiomaterials are extremely customizable through a variety of processing methods and chemical modifications, and are common in the clinical environment. This leaves researchers with many practical, clinically ready, and FDA-approved options. One very common application of polymers as nanomaterials is in the creation of therapeutic drug-loaded nanoparticles for sustained and targeted delivery to cells and tissue (Parveen, Misra, & Sahoo, 2012). Considering the process of proving a new biomaterial to the FDA as safe and effective is expensive and time-consuming, many researchers have begun extending the application of current biocompatible polymers already approved by the FDA for other medical devices for use in drug-loaded nanoparticle fabrication. For instance, polylactic acid (PLA), polyglycolic acid (PGA), poly(lactic-co-glycolic acid) (PLGA), and various prepolymer hydrogels including PEG-DA, GelMA, and methacrylate hyaluronic acid currently are generating huge interest from scientists to investigate their potentials for numerous regeneration applications due to their excellent biocompatibility, suitable mechanical properties, biodegradability, and ease of modification for different applications (Choi, Yong, Choi, & Cowie, 2019; Hann et al., 2019). A myriad of well-designed polymer-based nanoparticles loaded with growth factors or other therapeutics have been created for tissue engineering applications (Castro, Umanzor-Alvarez, Grace Zhang, & Keidar, 2012; Dhandayuthapani, Yoshida, Maekawa, & Kumar, 2011).

As we know, various transforming growth factors (*e.g.*, TGF- β 1 and TGF- β 3), and bone morphogenic proteins (*e.g.*, BMP-7, BMP-6, or BMP-2) have been shown to improve bone and cartilage regeneration (Bai, Li, Zhao, Duan, & Qu, 2011; Chim, Miller, Gliniak, & Alsberg, 2012; Cui, Zhu, Holmes, & Zhang, 2016; Kim, Erickson, Choudhury, Pleshko, & Mauck, 2012; Noel et al., 2004; Sekiya, Colter, & Prockop, 2001). However, for *in vivo* applications, these growth factors face ongoing issues related to short-term retention, quick half-life in circulation, and quick loss of biological activity *in vivo* even when administered at higher doses. For example, when delivered directly to tissue defect sites, they rapidly diffuse to adjacent tissues and lose their bioactivity, which limits their potential to promote prolonged tissue formation within a targeted site. In our laboratory, we have explored the use of coaxial electrospraying (Figure 1.4A) for highly efficient encapsulation of growth factors and therapeutics into a core-shell polymer nanosphere (Figure 1.4B) (Castro, O'Brien, & Zhang, 2014; Zhu, Masood, O'Brien, & Zhang, 2015). The coaxial electrospraying technique allows easy fabrication of a controllable core-shell nanosphere with intact biologically active growth factor within the core and polymeric outer shell. In addition, it enables the separation of organic and aqueous phases and thus incorporation of biologically active components such as growth factors into the aqueous phase without exposing them to harmful organic solvents. Due to these advantages, a coaxial needle is not just limited to the use for the electrospraying technique but also employed for various bioprinting techniques to fabricate artificial tissues, especially for vascularized constructs (Cui, Zhu, & Zhang, 2019).

**FIGURE 1.4**

(A) Graphical representation of a coaxial electrospinning technique for the manufacture of growth factor-encapsulated core-shell nanospheres. (B) TEM image of bone morphogenetic protein-2 encapsulated PDO nanospheres. (C) SEM images of 3D-printed scaffolds. The red circle and blue square indicate vascular channels with 500 μm diameter and bone scaffold with 200 μm pores. Scale bar, 200 μm . (D) A red autofluorescence image of a PLA scaffold modified with genipin. (E) SEM images of surface morphologies of the fabricated scaffolds (anti-vWF, green and OPN, red) (PLA—polylactic acid control, BC—bioactive nanocoating modified PLA, cBC—genipin modified PLA, BCG—bioactive nanocoating with growth factors, and cBCG—genipin crosslinked bioactive nanocoating with growth factors). Scale bars, 20 μm . (F) Immunofluorescence images of the vascularized bone formation with surface loading of growth factors and MSC-HUVEC cocultured in the dynamic culture environment. Scale bars, 100 μm .

(A, B) Images are adapted with permission from [Castro et al. \(2014\)](#). (C–F) Images are adapted with permission from [Cui et al. \(2016\)](#).

The selected polydioxanone (PDO), known commercially as PDS, is commonly used as an absorbable suture (Sakamoto, Kiyokawa, Rikimaru, Watanabe, & Nishi, 2012; Thomas, Zhang, & Vohra, 2009; Venclauskas, Grubinskas, Mocevicius, & Kiudelis, 2011). Excellent biocompatibility, mechanical properties as well as a slower degradation rate, render it ideal for controlled drug delivery and tissue engineering applications (Kalfa et al., 2010; Madurantakam et al., 2009; Smith et al., 2008; Wang, Chen, & Wang, 2010; Zhu, Dang, Wang, Wang, & Wang, 2011). Our results showed that the PDO nanospheres exhibited a much slower release of BMP-2 when compared to scaffolds blended with the growth factor, which contributed to the improved osteogenic performance of MSCs. The use of optimally regulated growth factors with biocompatible polymers can be incorporated into the fabrication of vascularized bone constructs by triggering osteogenesis and angiogenesis (Cui et al., 2016). Our laboratory has developed 3D-printed vascularized bone models with PLA (Figure 1.4C–E). While the 3D model with only PLA served as a control, PLA-based scaffolds with surface modification using consecutive adsorption of growth factors, such as BMP-2 and VEGF, to create gelatin and polylysine bioactive nanocoating layers were investigated. As a result, the scaffolds with surface loading of growth factors and coculture of MSCs and human umbilical vein endothelial cells (HUVECs) presented a much dense formation of vascularized bone (Figure 1.4F) (Cui et al., 2016).

1.2.3.2 Ceramic Nanobiomaterials and Ceramic-Polymer Nanocomposites

Ceramics are nonmetallic inorganic crystalline materials that express excellent cytocompatibility, mechanical properties, and possibly biodegradability in the physiological environment which makes them attractive for orthopedic and dental applications. Commonly investigated ceramics for bone regeneration can be classified into two categories: bioactive calcium phosphates [such as nHA and tricalcium phosphate (TCP)] or biopassive ceramics (such as alumina and zirconia) (Zhang, Chen, et al., 2008; Zhang, Ramsaywack, et al., 2008; Zhang, Sirivisoot, et al., 2008). As we know, 70% of the human bone matrix is composed of inorganic crystalline nHA, which is typically 20–80 nm long and 2–5 nm thick (Holmes et al., 2012; Zhang & Webster, 2009). Additionally, other components in the bone matrix [such as collagen and noncollagenous proteins (laminin, fibronectin, vitronectin)] are nanometer-scale in dimension. Therefore novel nanostructured ceramics, which mimic the nanostructure of natural bone, have become quite popular. These nanobiomaterials exhibit increased surface area and contribute improved surface roughness, surface wettability, and cytocompatibility (Zhang, Chen, et al., 2008; Zhang, Ramsaywack, et al., 2008; Zhang, Sirivisoot, et al., 2008) to tissue-engineered bone constructs. For example, nHA is commonly used in bone tissue engineering, due to its natural abundance in native bone tissue. In one study, nHA and bone marrow aspirate (BMA) were combined to form a paste for use in posterolateral fusion surgeries across 46 patients. When evaluated 12 months later, there was no difference between bone formation rates of iliac crest autografts (the gold standard in this surgery) and the nHA/BMA mixture (Robbins, Lauryssen, & Songer, 2017). nHA has been shown to not only improve osteoblast behavior (Webster et al., 2000) but also improve bone-marrow-derived MSCs behavior *in vitro* (Castro et al., 2014) and improve new bone formation *in vivo* (Chang, Wu, Mao, & Ding, 2001; Huber et al., 2006). Another highly studied ceramic material is zirconium dioxide or zirconia. It enhances fracture toughness in other ceramics. Kong et al. studied nHA-added zirconia–alumina nanocomposites in load-bearing orthopedic applications (Kong, Bae, Lee, Kim, & Kim, 2005). The nHA-added zirconia–alumina nanocomposites contained biphasic calcium phosphates of nHA/TCP and

had higher flexural strength than conventionally mixed nHA-added zirconia–alumina composites. The *in vitro* tests showed that the proliferation and differentiation of osteoblasts on this nanocomposite gradually increased as the amount of added nHA increased.

Although nHA and other ceramics are powerful materials when used alone, they can be made more versatile in combination with polymers to create a nanocomposite tissue-engineered scaffold. This approach can effectively allow for the fabrication of biomimetic physical and chemical gradients in bone, cartilage, and osteochondral tissue. In recent studies, the presence of nHA within PEG-DA- or GelMA-based scaffolds was proven to enhance osteogenic differentiation of MSCs (Hann et al., 2021; Zhou et al., 2016). Besides blending ceramics with polymer, a nanocomposite engineered scaffold with surface treatment, such as cold atmospheric plasma, was found to promote chondrogenesis of MSCs (Lee et al., 2020). Using osteochondral tissue as an example in detail, osteochondral defects, caused by osteoarthritis and trauma, present a common and serious clinical problem. They are notoriously difficult to regenerate due to complex inherent, stratified soft/hard tissue structure, poor cell mobility within the matrix, lack of vascular network, and limited local progenitor cells. Continuous gradients of proteins and collagen fibers are found throughout the cartilage zones and are essential for load transfer and directed cell behavior (Dormer, Singh, Wang, Berkland, & Detamore, 2010; Dormer et al., 2012; Zhang, Hu, & Athanasiou, 2009). The subchondral bone and calcified zone also contain a gradient of calcified ECM (nHA) (Zhang et al., 2012), which provides osteochondral structural integrity (Zhang, Hu, et al., 2009). Considering the unique graded structure of osteochondral tissue, ceramic nanoparticles can be embedded within a polymer or hydrogel scaffold to form a ceramic gradient originating in the rigid subchondral region and terminating with zero ceramic component in the articulating cartilage region of a scaffold (Khanarian, Jiang, Wan, Mow, & Lu, 2012). The stiffness of the native microenvironment provides an essential stimulus that helps to shepherd the phenotypic differentiation of pluripotent and multipotent stem cells, such as MSCs, in conjunction with chemical and other stimuli. Therefore implantable multiphasic scaffolds that leverage spatially controlled stiffness gradients, morphogenetic factors, and configurable geometries are being developed in an effort to direct the differentiation and phenotypic expression of bone-marrow–derived MSCs toward osteogenic and chondrogenic cell types in one construct (Chen et al., 2011; Wang et al., 2009; Wang, Li, et al., 2010). Several researchers also utilize biomimetic spatially controlled gradients (Eriskin, Kalyon, & Wang, 2008; Zhang, Hu, & Xu, 2005) with graded PCL/nHA composite fiber meshes. The fibrous meshes were subsequently seeded with mouse preosteoblast cells and, after a 4-week culture period, a deposited ECM was observed exhibiting gradations of collagen type I and calcium that were similar to the gradients that exist in the osteochondral site.

Similar to osteochondral tissue, the ligament is another highly integrated tissue, which must perform in a complex manner under a high-stress environment. To best simulate regeneration and repair of this environment, techniques employing two or more disparate biomaterials to create composite scaffolds with greatly improved mechanical and biochemical properties are prominent. In ligament tissue engineering, the fibrous nature of the natural tissue has inspired researchers to use electrospun scaffolds with a density gradient, similar to the nHA constituent in osteochondral tissue engineering, generating a stiffness gradient as along with a multimaterial approach (Kuo, Marturano, & Tuan, 2010; Samavedi, Olsen Horton, Guelcher, Goldstein, & Whittington, 2011). This multiphasic approach consisted of cospinning PCL with incorporated nHA and poly(ester urethane). The result was a graded scaffold that had both a biochemical gradient and a mechanical strength gradient that more accurately mimicked the natural ligament ECM (Samavedi et al., 2011).

1.3 3D NANO/MICROFABRICATION TECHNOLOGY FOR TISSUE REGENERATION

1.3.1 3D NANOFIBROUS AND NANOPOROUS SCAFFOLDS FOR TISSUE REGENERATION

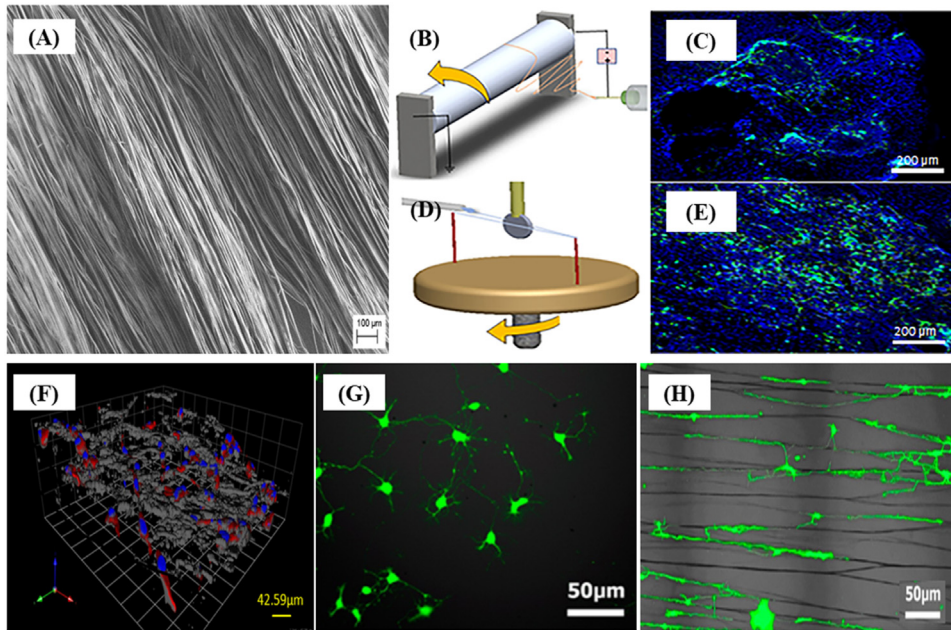
1.3.1.1 *Electrospun Nanofibrous Scaffolds for Tissue Regeneration*

To date, one of the most prominent nanofibrous scaffold fabrication methods is electrospinning. Similar to the aforementioned electrospraying technique, electrospinning utilizes the same equipment, but with a polymer of higher viscosity, allowing the stream not to break up and form droplets. It is a process by which a charged polymer is dissolved in a solvent, and exposed to a large voltage potential of several kilovolts as it is slowly pumped from a needle. The large electrical potential causes the fluid to be drawn out into a fine stream, which solidifies into fibers that can be dimensionally controlled by varying the viscosity, voltage potential, flow rate, and working distance between the needle and collector plate during fabrication. [Figure 1.5A](#) shows an electrospun highly aligned fibrous scaffold with conductive CNTs fabricated in our laboratory. This type of nanofibrous scaffold tends to be most effective when used for tissues with similar fibrous morphologies, and has been shown to be effective in many skin, musculoskeletal, and neural tissue regeneration applications ([Holmes et al., 2012](#); [Zhu, O'Brien, O'Brien, & Zhang, 2014](#)). Popularity for neural tissue engineering is due to the ability to create highly aligned nanofibrous scaffolds from many biocompatible polymeric materials. These scaffolds have been shown to more effectively promote neural outgrowth to bridge given defect sites ([Assmann et al., 2010](#)). According to the reported study by Mirzaei et al., stem cells seeded on electrospun carbon nanofibers that were derived from polyacrylonitrile exhibited great multidirectional stretchability and differentiation capability into targeting neural cells ([Mirzaei et al., 2016](#)). Our research also demonstrated electrospun carbon nanofibrous scaffolds that were acquired by the annealing approach to obtain an integrated network architecture for neural tissue engineering ([Zhu et al., 2018](#)). Due to the structural and biological integrity of the carbon nanofibrous scaffolds, it was observed that the proliferation of NSCs and neuronal differentiation activity was ideally enhanced under electrical stimulation after 7 days of culture.

Even though electrospinning normally creates thin constructs, the high surface area to volume ratio, nanometer feature size, and relative ease of fabrication make electrospun nanofibrous scaffolds beneficial for bone and cartilage tissue engineering, which have been thoroughly reviewed in our previous paper ([Holmes et al., 2012](#)). For example, Aclam et al. used electrospun PCL nanofibers and collagen type I to create an injectable scaffold that promotes bone regeneration. Briefly, they electrospun PCL fibers, combined them with a cell-laden collagen gel, and allowed the composite to cross-link naturally at physiologic conditions. After 21 days of culture, the injectable scaffolds showed increased total protein, alkaline phosphatase, and calcium concentration when compared to a pure collagen control ([Baylan et al., 2013](#)).

1.3.1.2 *Other 3D Nanofibrous/Nanoporous Scaffolds for Tissue Regeneration*

Besides nanofibrous scaffolds fabricated via electrospinning, other fabrication methods for nanofibrous or nanoporous scaffolds are also commonly utilized in tissue engineering. For example, one of the recently developed techniques called touch- and brush-spinning excludes the use of the

**FIGURE 1.5**

(A) SEM image of highly aligned electrospun scaffold with 0.5% CNTs. (B) Schematic illustration of electrospinning fabrication and (C) confocal image of differentiated NSC spreading on the electrospun scaffolds. Green: TUJ1; Blue: DAPI. (D) Schematic illustration of touch-spinning fabrication and (E) confocal image of differentiated NSC spreading on the touch-spun scaffolds. Green: TUJ1; Blue: DAPI. (F) Confocal microscopy image of MSC growth in a cold plasma modified nano bone scaffold. Blue represents cell nuclei stained by DAPI; Red represents cytoskeleton stained by Rhodamine-Phalloidin; Gray represents the porous nHA/chitosan scaffold. Confocal micrographs of neurons (green) cultured for 5 days on (G) the polystyrene substrate and (H) the parallel-aligned CNT yarns (black lines) substrate, respectively.

(A–E) Images are adapted with permission from [Asheghali et al. \(2020\)](#). (F) Image is adapted with permission from [Wang et al. \(2014\)](#). (G, H) Images are adapted with permission from [Fan et al. \(2012\)](#).

electric field ([Tokarev et al., 2015](#)). In contrast to electrospinning, the touch- and brush-spinning system uses a rotating rod on a spinneret to adhere to the polymer solution and a round hairbrush to collect nanofibers, respectively. Recently, our laboratory demonstrated and reported the study of nerve regeneration using touch-spun nanofibers ([Figure 1.5B–E](#)) ([Asheghali et al., 2020](#); [Lee et al., 2019](#)). The system allowed the fabrication of aligned PCL nanofibers with optimal crystallinity for neural structures, in which NSCs proliferated and differentiated for 8 days. On the other hand, for musculoskeletal tissue studies, conventional scaffold fabrication methods such as solvent casting and particle leaching ([Jiang et al., 2007](#); [Mikos et al., 1994](#)), and freeze drying ([Whang, Thomas, Healy, & Nuber, 1995](#)) have been widely used to fabricate 3D porous tissue scaffolds, which have been shown to influence cell functions and improve cartilage and bone regeneration ([Castro, Hacking, & Zhang, 2012](#); [Zhang, Hu, et al., 2009](#)). 3D porous hydrothermally treated nHA/chitosan

nanocomposite scaffolds have been fabricated through a freeze-drying method with cold plasma treatment (Wang et al., 2014). The results revealed that all nHA embedded, plasma modified chitosan scaffolds (Figure 1.5F) significantly enhanced MSC growth, migration and osteogenic differentiation *in vitro*. In addition, for ligament tissue regeneration, it has been shown that fibrous scaffolds employing natural silk are strong, relatively easy to work with, and display biomimetic amino acids on the surface of the material, increasing stem cell performance (Chen et al., 2012). In that study, Chen et al. capitalized on the strength of a macrofibrous knitted silk sponge scaffold coated with self-assembled RADA16 peptide nanofibers in the form of a nanofibrous mesh to increase cell performance (Chen et al., 2012). Scaffolds treated with RADA16 showed increased maximum tensile strength, collagen and glycosaminoglycan synthesis compared to bare controls. This synergistic effect of combining a microfibrous scaffold with nanomaterial coatings also illustrates the importance of biomimetic nanocomposites in tissue engineering.

Other, even more novel methods of scaffold fabrication are constantly being explored, Fan et al. were able to draw out superaligned CNT yarn and apply it to neural tissue engineering. The yarns described are not only biomimetic and able to direct neural growth (Fan et al., 2012) but also exist as a transition between traditional fabrication methods and customizable scaffold designs. One could imagine the customization of the weave of the yarn (Figure 1.5G and H) to the dimension of a neural defect in a particular subject, enabling researchers and medical professionals to adapt to the individual situation present in each animal model or human patient.

1.3.2 3D PRINTING OF NANOMATERIAL SCAFFOLDS FOR TISSUE REGENERATION

1.3.2.1 3D Printing Techniques for Tissue Regeneration

As an emerging 3D tissue manufacturing technique, 3D printing offers great precision and control of the architecture of a scaffold, and prints complicated structures which closely mirror biological tissues (Derby, 2012). 3D printing has become a driving force in the tissue engineering field with the advent of personalized medicine and the growing interest in complex tissue and organ regeneration (Catros et al., 2011, 2012; Cui, Breitenkamp, Finn, Lotz, & D'lima, 2012; Cui et al., 2017; Cui, Esworthy, et al., 2019; Detsch et al., 2011; Fedorovich, Schuurman, et al., 2012, 2012; Gruene et al., 2011; Hann et al., 2019; Hann, Cui, Nowicki, & Zhang, 2020; Holmes, Zhu, Li, Lee, & Zhang, 2015; Koch et al., 2012; Lee & Wu, 2012; Moon et al., 2010; Ovsianikov et al., 2010; Shim, Kim, Park, Park, & Cho, 2011; Song et al., 2010; Tasoglu & Demirci, 2013; Warnke et al., 2010; Zhou et al., 2020). The detailed information of 3D-printing techniques for tissue and organ regeneration was thoroughly reviewed in our previously published paper (Cui et al., 2017). Most 3D printers have micron resolutions far above the nanoscale, but are still a viable tool for nanomaterial fabrication and manipulation. Unlike traditional manufacturing techniques, 3D printing can deliver materials and cells to precise locations, resulting in constructs that can take advantage of computer-aided designed (CAD) and biomimetic morphology to create shapes that would be difficult or impossible to manufacture traditionally. All 3D-printing methods operate upon similar principles. To fabricate a solid object, a CAD model is first input to a program that parses the solid object into a stack of thin axial cross sections. These cross sections are then converted into directions that describe the movement of the effector in 3D space. The effector deposits material, solidifies resin, or otherwise performs an action that prints the CAD model itself and is

controlled to reproduce each cross-sectional slice delivered to the printer. Finally, all of the individual elements come together and the construct is serially printed from the bottom up.

It is important to note that 3D-printing techniques can print materials with or without incorporated cells, and each approach comes with various advantages and disadvantages. The major defining difference between the two is that bioprinting cells must maintain an environment that facilitates cellular survival and mitigates contamination or infection. 3D printing will be discussed at length later in this book, but briefly, several common methods (Figure 1.6) include inkjet bioprinting (Ferris et al., 2013), bioplotting (Fedorovich, Leeuwenburgh, Van Der Helm, Alblas, & Dhert, 2012), fused deposition modeling (Kundu, Shim, Jang, Kim, & Cho, 2015), selective laser sintering, and stereolithography (Lu, Mapili, Suhali, Chen, & Roy, 2006; Suri et al., 2011; Zhu et al., 2016). One of the advantages of 3D printing is to create custom-designed tissue constructs with complex internal architecture and biomimetic external architecture. As illustrated in Figure 1.7A, an MRI image of human osteochondral tissue defects is reconstructed into a unique CAD model. This patient-specific, osteochondral construct can be printed to perfectly integrate with the defect site and expedite tissue regeneration. Figure 1.7C–H shows that several custom-designed 3D PEG-DA-based hydrogel scaffolds with varying pore sizes were fabricated via a stereolithography printer (Figure 1.7B) in our laboratory. 3D-printed scaffolds for osteochondral

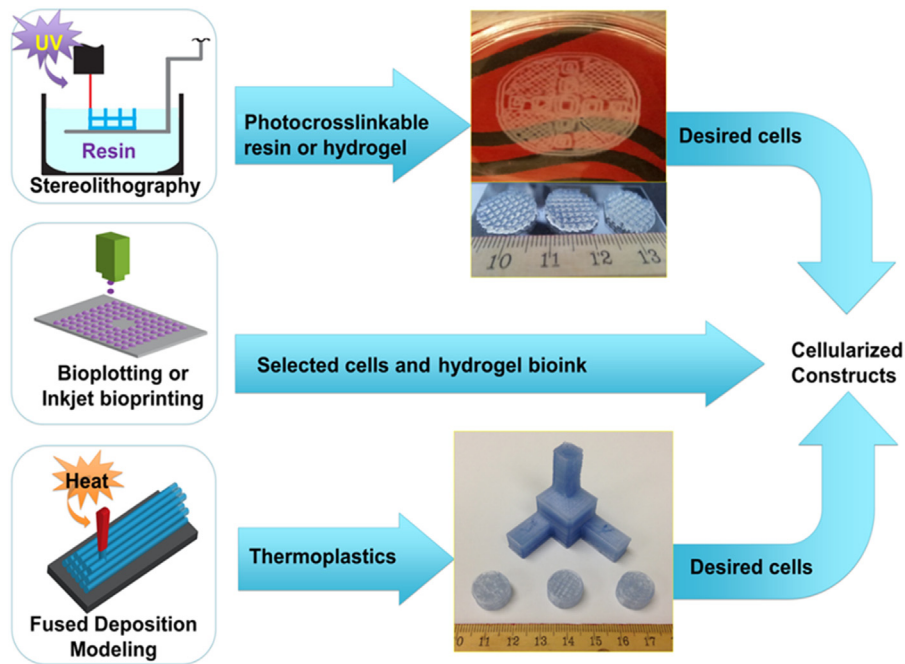


FIGURE 1.6

A schematic illustration of several typical 3D printing systems.

Image is adapted with permission from O'Brien, Holmes, Faucett, and Zhang (2015).

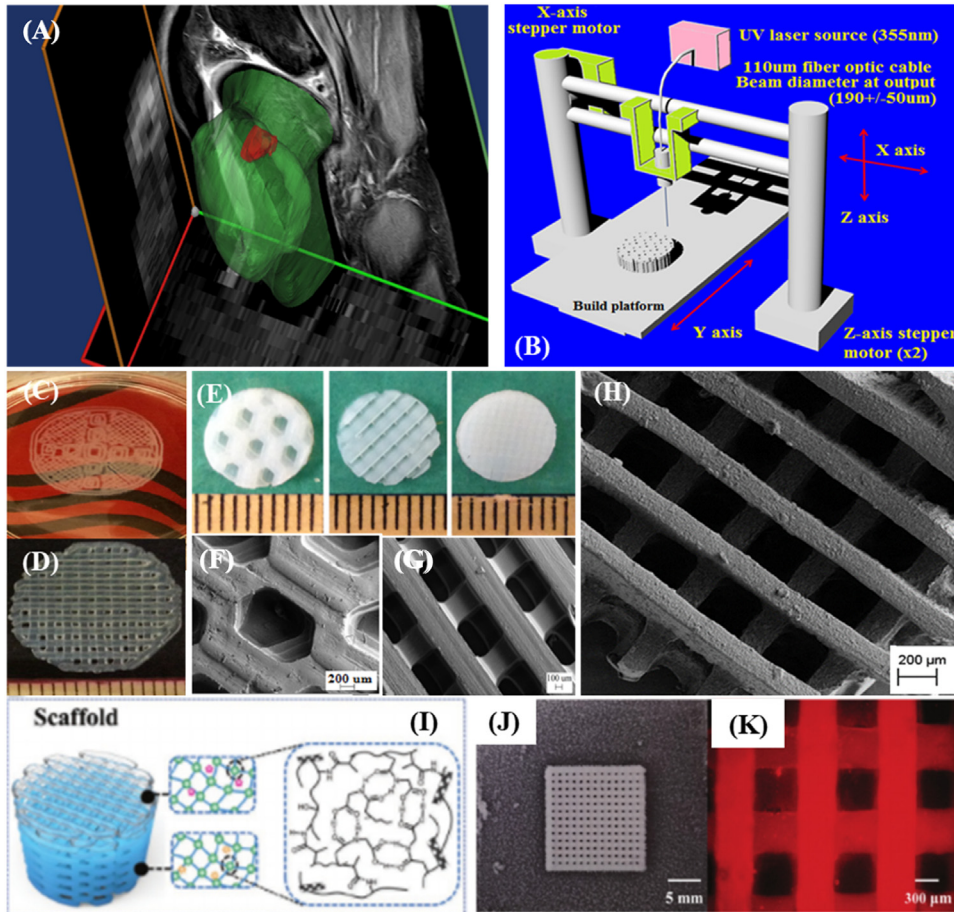
regeneration with the different compositions of bioinks including GelMA and poly(*N*-acryloyl 2-glycine) (PACG) also exhibit similar architectures (Figure 1.7I–K) (Gao et al., 2019).

A quickly growing fabrication method for more detailed structures is a technique called two-photon polymerization. Here two light sources are used: one to excite the material to an intermediate state and another to initiate crosslinking. Through this method, scaffolds can be created with feature sizes of $<10\text{ }\mu\text{m}$, allowing researchers to generate CAD models on a smaller scale, and print constructs in various specific geometries with high resolution. Femtosecond laser two-photon polymerization is one of the highest resolution 3D-printing technologies in use today. Initially, only commercially available polymers (Ovsianikov, Schlie, Ngezhahayo, Haverich, & Chichkov, 2007) could be printed, which may have lacked the necessary physical, chemical, and cell favorable characteristics needed for biomimetic tissue engineering. Some materials, such as 4,4'-bis(dimethylamino) benzophenone (SZ2080), can support cell growth and migration (Raimondi et al., 2013), and have effectively demonstrated that cells can react to microgeometries with pores varying from 10 to $30\text{ }\mu\text{m}$. Scaffolds with graded microporosity outperformed single-dimension microporous scaffolds, and cells tended to migrate to designed niches on the surface of the scaffold, as opposed to flat surfaces. The biocompatible PEG was used in a two-photon polymerization printer to produce engineered 3D scaffolds (Torgersen et al., 2012). This work focused on using two-photon polymerization in proximity to living tissue, and used a near-infrared 100 fs laser instead of a traditional, generally nonbiocompatible UV laser. With a specially developed photoinitiator customized for near-infrared wavelengths, researchers were able to print scaffolds approaching $300\text{ }\mu\text{m}^2$. Despite Raimondi and Torgersen's success, Torgersen also mentioned that the scaffolds were only $280\text{ }\mu\text{m} \times 280\text{ }\mu\text{m} \times 225\text{ }\mu\text{m}$, and Raimondi mentioned that on average 17 cells were found attached to the scaffolds after 6 days of *in vitro* culture. This serves to highlight the inherent limitations of the two-photon approach; particularly, a small overall scaffold-size that limits the scaffolds dimensions to a few hundred microns in each dimension, far below clinical relevance.

1.3.2.2 3D Printing of Nanomaterial Scaffolds for Tissue Regeneration

Nanomaterials for 3D printing have been recently developed. One nanobiomaterial in use is bacterial nanocellulose (BNC). BNC is a naturally occurring nanomaterial synthesized by bacteria that has been used, in this case, with a 3D printing system to produce patient-specific auricular constructs that closely match natural geometries (Nimeskern et al., 2013). This material is nanofibrous and promotes adhesion of endothelial and NIH/3T3 cell lines (Fu, Zhou, Zhang, & Yang, 2013), making it an excellent potential biomaterial for the 3D fabrication of chondrogenic scaffolds. Other nanomaterials and nanocomposite biomaterials are being developed for use in tissue engineering, and many need an only simple modification to be compatible with a number of 3D printing modalities.

As an example of nanobiomaterial utilization, our laboratory has developed a table-top stereolithography setup using a UV laser as the excitation source (Figure 1.7B). It has been used to crosslink nHA impregnated photocrosslinkable PEG-DA hydrogel into 3D osteochondral scaffolds (Figure 1.7H). These scaffolds are unique in that they have a biologically inspired gradient of nHA to guide MSC differentiation to osteogenic and chondrogenic lineages within the same scaffold. This printer has additionally been used to print graphene nanoplatelets suspended in PEG-DA hydrogel into 3D scaffolds for neural regeneration. Both examples showcase the potential of 3D printing to incorporate nanomaterials and traditional biomaterials within the same scaffold to achieve nanoscale features, but some printers attempt to create even higher resolution constructs without the addition of nanomaterials.

**FIGURE 1.7**

(A) An MRI image of an osteochondral defect (labeled as red color) in the human knee joint. (B) Schematic illustration of a tabletop stereolithography 3D bioprinter. (C–E) 3D-printed PEG-DA hydrogel scaffolds with varied designs. (F and G) SEM images of 3D-bioprinted PEG-DA scaffolds with hexagonal and square pores; and (H) with nHA particles for osteochondral regeneration. (I) A schematic illustration of hydrogel bonding interactions between PACG and GelMA after UV crosslinking. (J) A photo image of 3D-printed PACG-GelMA hydrogel scaffolds. (K) A microscope image of PACG-GelMA hydrogel scaffolds when reaching swelling equilibrium in water.

(A–H) Images are adapted with permission from [Castro, O'Brien, and Zhang \(2015\)](#); [Holmes et al. \(2015\)](#); [O'Brien et al. \(2015\)](#).

(I–K) Images are adapted with permission from [Gao et al., \(2019\)](#).

Another nanomaterial used in 3D printing has been the bioceramic TCP. TCP can be processed into a fine powder ([Tarafder, Balla, Davies, Bandyopadhyay, & Bose, 2013](#)) and applied in a novel 3D sintering method that uses directed microwaves to selectively heat and sinter the fine powder particles together, layer by layer, into a 3D scaffold ([Tarafder et al., 2013](#); [Wagner, Jones, Zhou, &](#)

Bhaduri, 2013). Microwave-sintered TCP scaffolds exhibited an increase in compressive strength and more optimal microporosity to macroporosity ratio when compared to scaffolds fabricated with traditional laser energy sources. Furthermore, the microwave-sintered scaffolds increased the formation of new bone *in vivo* when compared to constructs fabricated through conventional sintering (Tarafder et al., 2013). Separately, a magnetic-based approach for engineering cartilage tissues has also been reported recently. Zlotnick et al. developed a system where naturally diamagnetic objects, such as polystyrene beads, delivery microcapsules, and cells, can be positioned in polymer-based 3D photocrosslinkable hydrogels to efficiently design multidirectional 3D patterns after a short exposure to a magnetic field (Zlotnick et al., 2020).

Because it is known that nanobiomaterials are integral to eliciting the optimal response from many cell lines, it may seem beneficial to target nanoscale structures in future 3D printing systems. However, the challenges involved in realizing such precise designs can make fabricating objects with nanoscale precision somewhat impractical. Researchers have employed everything from dynamic masking type procedures to high resolution digital micromirror array photocuring to highly tuned two-photon femtosecond laser-based printing. Although these methods are extremely high resolution, the speed at which structures can be fabricated is on the order of hours, and is still limited to a maximum build envelope of several hundred microns. These two major factors limit the potential efficacy of these types of systems as clinical tools, in favor of fabrication technology that can rapidly produce defect-sized constructs in a time-efficient manner. Although many challenges are ahead, many are hopeful that with the right combination of hardware, software, and novel nanobiomaterials, advanced nanoscale 3D printing can one day be realized and implemented. More detailed information about integrating 3D-bioprinting techniques with nanomaterials for complex tissue and organ regeneration can be referred to (Cui et al., 2017; Hann et al., 2019; O'Brien et al., 2015).

1.4 CONCLUSION AND FUTURE DIRECTIONS

Current tissue engineering methods can readily create materials, structures, and scaffolds that can support one or a few cell types *in vitro*, but *in vivo* many challenges exist that limit the efficacy of current, single tissue designs. There will naturally be differences between living tissue and the implanted remedy, so researchers must take this into account and allow for proper integration without major mechanical and biochemical disparities. Furthermore, many injuries that would benefit from a tissue-engineered solution, such as osteochondral, ligament, and nerve damage, occur at the interface of two or more tissue types. This means a fully successful implant must simultaneously support the growth of different cell types and tissues, each with specific mechanical properties, chemical gradients, cell populations, and specific geometric constraints incorporated within the scaffold design. The myriad of complex design constraints limits the effectiveness of many current methods, especially when attempting to regenerate clinically relevant injuries, organs, and other complex tissues and tissue interfaces.

To address the inherent limitations and requirements posed by the increased complexity of interfacial tissue engineering, nanotechnologies (such as nanobiomaterials) are increasingly being utilized. Nanobiomaterials clearly have played an integral role in tissue engineering, and will continue to be an important design consideration for future work. The beauty of incorporating nanomaterials into tissue-engineered constructs is the versatility they contribute almost intrinsically. Many

nanomaterials add similarly vast improvements to constructs they are incorporated within. The cutting edge of tissue engineering and regenerative medicine research is moving toward customizing therapies to individual patients and individual situations. For nanomaterials to be integrated into a patient-specific scaffold, manufacturing techniques need to be employed to allow for further micro- and macroscale customization; exactly what 3D printing excels at.

Now, with the introduction of 3D printing in the tissue engineering field, researchers can begin to experience the benefits of having truly unique solutions to problems not easily solved with traditional fabrication techniques. A robust hobbyist community and open source movement, RepRap, has been driving down the cost of implementation of many 3D-printing technologies, making them available for researchers and clinicians worldwide. This has made 3D-printing technology readily available to the research community, and thus 3D bioprinting has been advancing at an exciting pace. The successful implementation of a scaffold with complex requirements, including the utilization of multiple disparate materials and several nanomaterial constituents into a patient-specific geometry with highly defined internal microgeometry, becomes surmountable. By incorporating multiple cell types, biomaterials, and nanomaterials in specific, biomimetic geometries, tissue engineers can expect to develop truly revolutionary medical devices, therapies and treatments, and potentially usher in a new age of regenerative medicine.

ACKNOWLEDGMENTS

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QUESTIONS

- Q1. What are the key properties of nanobiomaterials that make them good candidates for tissue engineering applications?
- Q2. What are the typical growth factors used for bone and cartilage regeneration and their limitations to be used *in vivo*?
- Q3. What is the most common technique for fabricating the nanofibrous tissue scaffold?
- Q4. What are the benefits of using 3D printing for complex tissue and organ regeneration?
- Q5. Why are nanomaterials investigated as 3D printing inks?
- Q6. What are the current challenges and future directions of tissue engineering?

References

- Abarrategi, A., Gutierrez, M. C., Moreno-Vicente, C., Hortiguera, M. J., Ramos, V., Lopez-Lacomba, J. L., . . . Del Monte, F. (2008). Multiwall carbon nanotube scaffolds for tissue engineering purposes. *Biomaterials*, 29, 94–102.
- Aida, T., Meijer, E. W., & Stupp, S. I. (2012). Functional supramolecular polymers. *Science (New York, N.Y.)*, 335, 813–817.

- Akhavan, O., & Ghaderi, E. (2013). Differentiation of human neural stem cells into neural networks on graphene nanogrids. *Journal of Materials Chemistry B*, 1, 6291–6301.
- Asheghali, D., Lee, S.-J., Furchner, A., Gruz, A., Larson, S., Tokarev, A., ... Zhang, L. G. (2020). Enhanced neuronal differentiation of neural stem cells with mechanically enhanced touch-spun nanofibrous scaffolds. *Nanomedicine: Nanotechnology, Biology and Medicine*, 24, 102152.
- Assmann, U., Szentivanyi, A., Stark, Y., Scheper, T., Berski, S., Drager, G., & Schuster, R. H. (2010). Fiber scaffolds of polysialic acid via electrospinning for peripheral nerve regeneration. *Journal of Materials Science. Materials in Medicine*, 21, 2115–2124.
- Bai, X., Li, G., Zhao, C., Duan, H., & Qu, F. (2011). BMP7 induces the differentiation of bone marrow-derived mesenchymal cells into chondrocytes. *Medical & Biological Engineering & Computing*, 49, 687–692.
- Baylan, N., Bhat, S., Ditto, M., Lawrence, J. G., Lecka-Czernik, B., & Yildirim-Ayan, E. (2013). Polycaprolactone nanofiber interspersed collagen type-I scaffold for bone regeneration: A unique injectable osteogenic scaffold. *Biomedical Materials (Bristol, England)*, 8, 045011.
- Biggs, M. J., Richards, R. G., Gadegaard, N., Wilkinson, C. D., & Dalby, M. J. (2007). Regulation of implant surface cell adhesion: Characterization and quantification of S-phase primary osteoblast adhesions on biomimetic nanoscale substrates. *Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society*, 25, 273–282.
- Castro, N., Umazor-Alvarez, J., Grace Zhang, L., & Keidar, M. (2012). Nanobiotechnology and nanostructured therapeutic delivery systems. *Recent Patents on Biomedical Engineering*, 5, 29–40.
- Castro, N. J., Hacking, S. A., & Zhang, L. G. (2012). Recent progress in interfacial tissue engineering approaches for osteochondral defects. *Annals of Biomedical Engineering*, 40, 1628–1640.
- Castro, N. J., O'Brien, C., & Zhang, L. G. (2014). Biomimetic biphasic 3D nanocomposite scaffold for osteochondral regeneration. *AIChE Journal*, 60, 432–442.
- Castro, N. J., O'Brien, J., & Zhang, L. G. (2015). Integrating biologically inspired nanomaterials and table-top stereolithography for 3D printed biomimetic osteochondral scaffolds. *Nanoscale*, 7(33), 14010–14022. Available from <https://doi.org/10.1039/C5NR03425F>.
- Catros, S., Fricain, J. C., Guillotin, B., Pippenger, B., Bareille, R., Remy, M., ... Guillemot, F. (2011). Laser-assisted bioprinting for creating on-demand patterns of human osteoprogenitor cells and nano-hydroxyapatite. *Biofabrication*, 3, 025001.
- Catros, S., Guillemot, F., Nandakumar, A., Ziane, S., Moroni, L., Habibovic, P., ... Fricain, J. C. (2012). Layer-by-layer tissue microfabrication supports cell proliferation *in vitro* and *in vivo*. *Tissue Engineering. Part C, Methods*, 18, 62–70.
- Chang, C. K., Wu, J. S., Mao, D. L., & Ding, C. X. (2001). Mechanical and histological evaluations of hydroxyapatite-coated and noncoated Ti6Al4V implants in tibia bone. *Journal of Biomedical Materials Research*, 56, 17–23.
- Chen, J., Chen, H., Li, P., Diao, H., Zhu, S., Dong, L., ... Zhang, J. (2011). Simultaneous regeneration of articular cartilage and subchondral bone *in vivo* using MSCs induced by a spatially controlled gene delivery system in bilayered integrated scaffolds. *Biomaterials*, 32, 4793–4805.
- Chen, K., Sahoo, S., He, P., Ng, K. S., Toh, S. L., & Goh, J. C. (2012). A hybrid silk/RADA-based fibrous scaffold with triple hierarchy for ligament regeneration. *Tissue Engineering. Part A*, 18, 1399–1409.
- Childs, A., Hemraz, U. D., Castro, N. J., Fenniri, H., & Zhang, L. G. (2013). Novel biologically-inspired rosette nanotube PLLA scaffolds for improving human mesenchymal stem cell chondrogenic differentiation. *Biomedical Materials (Bristol, England)*, 8, 065003.
- Chim, H., Miller, E., Gliniak, C., & Alsberg, E. (2012). Stromal-cell-derived factor (SDF) 1- α in combination with BMP-2 and TGF- β 1 induces site-directed cell homing and osteogenic and chondrogenic differentiation for tissue engineering without the requirement for cell seeding. *Cell and Tissue Research*, 350, 89–94.

- Choi, J. R., Yong, K. W., Choi, J. Y., & Cowie, A. C. (2019). Recent advances in photo-crosslinkable hydrogels for biomedical applications. *Biotechniques*, 66, 40–53.
- Choi, J. R., Yong, K. W., Choi, J. Y., Nilghaz, A., Lin, Y., Xu, J., & Lu, X. (2018). Black phosphorus and its biomedical applications. *Theranostics*, 8, 1005.
- Chopra, K., Mummery, P. M., Derby, B., & Gough, J. E. (2012). Gel-cast glass-ceramic tissue scaffolds of controlled architecture produced via stereolithography of moulds. *Biofabrication*, 4, 045002.
- Crowder, S. W., Prasai, D., Rath, R., Balikov, D. A., Bae, H., Bolotin, K. I., & Sung, H. J. (2013). Three-dimensional graphene foams promote osteogenic differentiation of human mesenchymal stem cells. *Nanoscale*, 5, 4171–4176.
- Cui, H., Esworthy, T., Zhou, X., Hann, S. Y., Glazer, R. I., Li, R., & Zhang, L. G. (2019). Engineering a novel 3D printed vascularized tissue model for investigating breast cancer metastasis to bone. *Advanced Healthcare Materials*. Available from <https://doi.org/10.1002/adhm.2019009>.
- Cui, H., Miao, S., Esworthy, T., Zhou, X., Lee, S. J., Liu, C., . . . Zhang, L. G. (2018). 3D bioprinting for cardiovascular regeneration and pharmacology. *Advanced Drug Delivery Reviews*, 132, 252–269.
- Cui, H., Nowicki, M., Fisher, J. P., & Zhang, L. G. (2017). 3D bioprinting for organ regeneration. *Advanced Healthcare Materials*, 6. Available from <https://doi.org/10.1002/adhm.201601118>.
- Cui, H., Zhu, W., & Zhang, L. G. (2019). Coaxial needle for fabricating a multi scale, multi layer blood vessel or vascular network employing 3D bioprinting. *Google patents US20190008998A1*.
- Cui, H., Zhu, W., Holmes, B., & Zhang, L. G. (2016). Biologically inspired smart release system based on 3D bioprinted perfused scaffold for vascularized tissue regeneration. *Advanced Science*, 3, 1600058. Available from <https://doi.org/10.1002/advs.201600058>.
- Cui, X., Breitenkamp, K., Finn, M. G., Lotz, M., & D'lima, D. D. (2012). Direct human cartilage repair using three-dimensional bioprinting technology. *Tissue Engineering. Part A*, 18, 1304–1312.
- Derby, B. (2012). Printing and prototyping of tissues and scaffolds. *Science (New York, NY)*, 338, 921–926.
- Detsch, R., Schaefer, S., Deisinger, U., Ziegler, G., Seitz, H., & Leukers, B. (2011). *In vitro*: Osteoclastic activity studies on surfaces of 3D printed calcium phosphate scaffolds. *Journal of Biomaterials Applications*, 26, 359–380.
- Dhandayuthapani, B., Yoshida, Y., Maekawa, T., & Kumar, D. S. (2011). Polymeric scaffolds in tissue engineering application: A review. *International Journal of Polymer Science*, 2011. Available from <https://doi.org/10.1155/2011/290602>.
- Dormer, N. H., Singh, M., Wang, L., Berkland, C. J., & Detamore, M. S. (2010). Osteochondral interface tissue engineering using macroscopic gradients of bioactive signals. *Annals of Biomedical Engineering*, 38, 2167–2182.
- Dormer, N. H., Singh, M., Zhao, L., Mohan, N., Berkland, C. J., & Detamore, M. S. (2012). Osteochondral interface regeneration of the rabbit knee with macroscopic gradients of bioactive signals. *Journal of Biomedical Materials Research. Part A*, 100, 162–170.
- Elias, K. L., Price, R. L., & Webster, T. J. (2002). Enhanced functions of osteoblasts on nanometer diameter carbon fibers. *Biomaterials*, 23, 3279–3287.
- Endo, M. (1988). Grow carbon fibers in the vapor phase. *Chemtech*, 18, 568–576.
- Engler, A. J., Sen, S., Sweeney, H. L., & Discher, D. E. (2006). Matrix elasticity directs stem cell lineage specification. *Cell*, 126, 677–689.
- Eriskien, C., Kalyon, D. M., & Wang, H. (2008). Functionally graded electrospun polycaprolactone and beta-tricalcium phosphate nanocomposites for tissue engineering applications. *Biomaterials*, 29, 4065–4073.
- Fan, L., Feng, C., Zhao, W., Qian, L., Wang, Y., & Li, Y. (2012). Directional neurite outgrowth on superaligned carbon nanotube yarn patterned substrate. *Nano Letters*, 12, 3668–3673.

- Fedorovich, N. E., Leeuwenburgh, S. C., Van Der Helm, Y. J., Alblas, J., & Dhert, W. J. (2012). The osteoinductive potential of printable, cell-laden hydrogel-ceramic composites. *Journal of Biomedical Materials Research. Part A*, 100, 2412–2420.
- Fedorovich, N. E., Schuurman, W., Wijnberg, H. M., Prins, H. J., Van Weeren, P. R., Malda, J., ... Dhert, W. J. (2012). Biofabrication of osteochondral tissue equivalents by printing topologically defined, cell-laden hydrogel scaffolds. *Tissue Engineering. Part C, Methods*, 18, 33–44.
- Ferris, C. J., Gilmore, K. J., Beirne, S., McCallum, D., Wallace, G. G., & In Het panhuis, M. (2013). Bio-ink for on-demand printing of living cells. *Biomaterials Science*, 1, 224.
- Fine, E., Zhang, L., Fenniri, H., & Webster, T. J. (2009). Enhanced endothelial cell functions on rosette nanotube-coated titanium vascular stents. *International Journal of Nanomedicine*, 4, 91–97.
- Florine, E. M., Miller, R. E., Porter, R. M., Evans, C. H., Kurz, B., & Grodzinsky, A. J. (2013). Effects of dexamethasone on mesenchymal stromal cell chondrogenesis and aggrecanase activity: Comparison of agarose and self-assembling peptide scaffolds. *Cartilage*, 4, 63–74.
- Frieden, E. (1972). The chemical elements of life. *Scientific American*, 227, 52–60.
- Fu, L. N., Zhou, P., Zhang, S. M., & Yang, G. (2013). Evaluation of bacterial nanocellulose-based uniform wound dressing for large area skin transplantation. *Materials Science & Engineering C-Materials for Biological Applications*, 33, 2995–3000.
- Gacem, K., Retrouvey, J. M., Chabi, D., Filoramo, A., Zhao, W., Klein, J. O., & Derycke, V. (2013). Neuromorphic function learning with carbon nanotube based synapses. *Nanotechnology*, 24, 384013.
- Gao, F., Xu, Z., Liang, Q., Li, H., Peng, L., Wu, M., ... Liu, W. (2019). Osteochondral regeneration with 3D-printed biodegradable high-strength supramolecular polymer reinforced-gelatin hydrogel scaffolds. *Advanced Science*, 6, 1900867.
- Gelain, F., Bottai, D., Vescovi, A., & Zhang, S. (2006). Designer self-assembling peptide nanofiber scaffolds for adult mouse neural stem cell 3-dimensional cultures. *PLoS One*, 1, e119.
- Gruene, M., Pflaum, M., Hess, C., Diamantouros, S., Schlie, S., Deiwick, A., ... Chichkov, B. (2011). Laser printing of three-dimensional multicellular arrays for studies of cell-cell and cell-environment interactions. *Tissue Engineering. Part C, Methods*, 17, 973–982.
- Hann, S. Y., Cui, H., Esworthy, T., Miao, S., Zhou, X., Lee, S.-J., ... Zhang, L. G. (2019). Recent advances in 3D printing: Vascular network for tissue and organ regeneration. *Translational Research*, 211, 46–63.
- Hann, S. Y., Cui, H., Esworthy, T., Zhou, X., Lee, S.-J., Plesniak, M. W., & Zhang, L. G. (2021). Dual 3D printing for vascularized bone tissue regeneration. *Acta Biomaterialia*, 123, 263–274.
- Hann, S. Y., Cui, H., Nowicki, M., & Zhang, L. G. (2020). 4D printing soft robotics for biomedical applications. *Additive Manufacturing*, 36, 101567.
- Harrison, B. S., & Atala, A. (2007). Carbon nanotube applications for tissue engineering. *Biomaterials*, 28, 344–353.
- Hartgerink, J. D., Beniash, E., & Stupp, S. I. (2001). Self-assembly and mineralization of peptide-amphiphile nanofibers. *Science (New York, N.Y.)*, 294, 1684–1688.
- Hauser, C. A., & Zhang, S. (2010). Designer self-assembling peptide nanofiber biological materials. *Chemical Society Reviews*, 39, 2780–2790.
- Holmes, B., Castro, N. J., Li, J., Keidar, M., & Zhang, L. G. (2013). Enhanced human bone marrow mesenchymal stem cell functions in novel 3D cartilage scaffolds with hydrogen treated multi-walled carbon nanotubes. *Nanotechnology*, 24, 365102.
- Holmes, B., Castro, N. J., Zhang, L. G., & Zussman, E. (2012). Electrospun fibrous scaffolds for bone and cartilage tissue generation: Recent progress and future developments. *Tissue Engineering. Part B, Reviews*, 18, 478–486.

- Holmes, B., Fang, X., Zarate, A., Keidar, M., & Zhang, L. G. (2016). Enhanced human bone marrow mesenchymal stem cell chondrogenic differentiation in electrospun constructs with carbon nanomaterials. *Carbon*, 97, 1–13.
- Holmes, B., Zhu, W., Li, J., Lee, J. D., & Zhang, L. G. (2015). Development of biomimetic 3D printed scaffolds for osteochondral regeneration. *Tissue Engineering Part A*, 21, 403–415.
- Hosseinkhani, H., Hosseinkhani, M., Tian, F., Kobayashi, H., & Tabata, Y. (2006). Osteogenic differentiation of mesenchymal stem cells in self-assembled peptide-amphiphile nanofibers. *Biomaterials*, 27, 4079–4086.
- Huber, F. X., Belyaev, O., Hillmeier, J., Kock, H. J., Huber, C., Meeder, P. J., & Berger, I. (2006). First histological observations on the incorporation of a novel nanocrystalline hydroxyapatite paste OSTIM in human cancellous bone. *BMC Musculoskeletal Disorders*, 7, 50.
- Huebsch, N., & Mooney, D. J. (2009). Inspiration and application in the evolution of biomaterials. *Nature*, 462, 426–432.
- Im, O., Li, J., Wang, M., Zhang, L. G., & Keidar, M. (2012). Biomimetic three-dimensional nanocrystalline hydroxyapatite and magnetically synthesized single-walled carbon nanotube chitosan nanocomposite for bone regeneration. *International Journal of Nanomedicine*, 7, 2087–2099.
- Jakus, A. E., Secor, E. B., Rutz, A. L., Jordan, S. W., Hersam, M. C., & Shah, R. N. (2015). Three-dimensional printing of high-content graphene scaffolds for electronic and biomedical applications. *ACS Nano*, 9, 4636–4648.
- Jang, M. J., Namgung, S., Hong, S., & Nam, Y. (2010). Directional neurite growth using carbon nanotube patterned substrates as a biomimetic cue. *Nanotechnology*, 21, 235102.
- Jiang, C. C., Chiang, H., Liao, C. J., Lin, Y. J., Kuo, T. F., Shieh, C. S., ... Tuan, R. S. (2007). Repair of porcine articular cartilage defect with a biphasic osteochondral composite. *Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society*, 25, 1277–1290.
- Jung, S. H., Song, W., Lee, S. I., Kim, Y., Cha, M. J., Kim, S. H., ... Park, C. Y. (2013). Synthesis of graphene and carbon nanotubes hybrid nanostructures and their electrical properties. *Journal of Nanoscience and Nanotechnology*, 13, 6730–6734.
- Kalfa, D., Bel, A., Chen-Tournoux, A., Della Martina, A., Rochereau, P., Coz, C., ... Menasche, P. (2010). A polydioxanone electrospun valved patch to replace the right ventricular outflow tract in a growing lamb model. *Biomaterials*, 31, 4056–4063.
- Karbasi, S., & Alizadeh, Z. M. (2017). Effects of multi-wall carbon nanotubes on structural and mechanical properties of poly(3-hydroxybutyrate)/chitosan electrospun scaffolds for cartilage tissue engineering. *Bulletin of Materials Science*, 40, 1247–1253.
- Khanarian, N. T., Jiang, J., Wan, L. Q., Mow, V. C., & Lu, H. H. (2012). A hydrogel-mineral composite scaffold for osteochondral interface tissue engineering. *Tissue Engineering. Part A*, 18, 533–545.
- Kim, M., Erickson, I. E., Choudhury, M., Pleshko, N., & Mauck, R. L. (2012). Transient exposure to TGF-beta3 improves the functional chondrogenesis of MSC-laden hyaluronic acid hydrogels. *Journal of the Mechanical Behavior of Biomedical Materials*, 11, 92–101.
- Kim, Y., Hayashi, T., Endo, M., & Dresselhaus, M. (2013). Carbon nanofibers. In R. Vajtai (Ed.), *Springer handbook of nanomaterials*. Berlin, Heidelberg: Springer.
- Kim, Y. G., Kim, J. W., Pyeon, H. J., Hyun, J. K., Hwang, J. Y., Choi, S. J., ... Lee, Y. I. (2014). Differential stimulation of neurotrophin release by the biocompatible nano-material (carbon nanotube) in primary cultured neurons. *Journal of Biomaterials Applications*, 28, 790–797.
- Koch, L., Deiwick, A., Schlie, S., Michael, S., Gruene, M., Coger, V., ... Chichkov, B. (2012). Skin tissue generation by laser cell printing. *Biotechnology and Bioengineering*, 109, 1855–1863.
- Kong, Y. M., Bae, C. J., Lee, S. H., Kim, H. W., & Kim, H. E. (2005). Improvement in biocompatibility of $\text{ZrO}_2\text{-Al}_2\text{O}_3$ nano-composite by addition of HA. *Biomaterials*, 26, 509–517.

- Kundu, J., Shim, J. H., Jang, J., Kim, S. W., & Cho, D. W. (2015). An additive manufacturing-based PCL-alginate-chondrocyte bioprinted scaffold for cartilage tissue engineering. *Journal of Tissue Engineering and Regenerative Medicine*, 9, 1286–1297.
- Kuo, C. K., Marturano, J. E., & Tuan, R. S. (2010). Novel strategies in tendon and ligament tissue engineering: Advanced biomaterials and regeneration motifs. *Sports Medicine, Arthroscopy, Rehabilitation, Therapy and Technology*, 2, 20.
- Lan, F., & Li, G. (2013). Direct observation of hole transfer from semiconducting polymer to carbon nanotubes. *Nano Letters*, 13, 2086–2091.
- Lee, J. E., Khang, D., Kim, Y. E., & Webster, T. J. (2006). Stem cell impregnated carbon nanofibers/nanotubes for healing damaged neural tissue. *MRS Proceedings*, 915, 17–22.
- Lee, M., & Wu, B. M. (2012). Recent advances in 3D printing of tissue engineering scaffolds. *Methods in Molecular Biology*, 868, 257–267.
- Lee, S.-J., Asheghali, D., Blevins, B., Timsina, R., Esworthy, T., Zhou, X., ... Tokarev, A. (2019). Touch-spun nanofibers for nerve regeneration. *ACS Applied Materials & Interfaces*, 12, 2067–2075.
- Lee, S.-J., Yan, D., Zhou, X., Cui, H., Esworthy, T., Hann, S. Y., ... Zhang, L. G. (2020). Integrating cold atmospheric plasma with 3D printed bioactive nanocomposite scaffold for cartilage regeneration. *Materials Science and Engineering: C*, 111, 110844.
- Lee, S.-J., Zhu, W., Nowicki, M., Lee, G., Heo, D. N., Kim, J., ... Zhang, L. G. (2018). 3D printing nano conductive multi-walled carbon nanotube scaffolds for nerve regeneration. *Journal of Neural Engineering*, 15, 016018.
- Li, Y.-L., Kinloch, I. A., & Windle, A. H. (2004). Direct spinning of carbon nanotube fibers from chemical vapor deposition synthesis. *Science (New York, NY)*, 304, 276–278.
- Liedtke, W., Yeo, M., Zhang, H., Wang, Y., Gignac, M., Miller, S., ... Liu, J. (2013). Highly conductive carbon nanotube matrix accelerates developmental chloride extrusion in central nervous system neurons by increased expression of chloride transporter KCC2. *Small*, 9, 1066–1075.
- Liu, X., Miller, A. L., Park, S., George, M. N., Waletzki, B. E., Xu, H., ... Lu, L. (2019). Two-dimensional black phosphorus and graphene oxide nanosheets synergistically enhance cell proliferation and osteogenesis on 3D printed scaffolds. *ACS Applied Materials & Interfaces*, 11, 23558–23572.
- Lu, Y., Mapili, G., Suhali, G., Chen, S., & Roy, K. (2006). A digital micro-mirror device-based system for the microfabrication of complex, spatially patterned tissue engineering scaffolds. *Journal of Biomedical Materials Research Part A*, 77, 396–405.
- Madurantakam, P. A., Rodriguez, I. A., Cost, C. P., Viswanathan, R., Simpson, D. G., Beckman, M. J., ... Bowlin, G. L. (2009). Multiple factor interactions in biomimetic mineralization of electrospun scaffolds. *Biomaterials*, 30, 5456–5464.
- Martinelli, V., Cellot, G., Fabbro, A., Bosi, S., Mestroni, L., & Ballerini, L. (2013). Improving cardiac myocytes performance by carbon nanotubes platforms. *Frontiers in Physiology*, 4, 239.
- Martinelli, V., Cellot, G., Toma, F. M., Long, C. S., Caldwell, J. H., Zentilin, L., ... Mestroni, L. (2012). Carbon nanotubes promote growth and spontaneous electrical activity in cultured cardiac myocytes. *Nano Letters*, 12, 1831–1838.
- Mikos, A. G., Thorsen, A. J., Czerwonka, L. A., Bao, Y., Langer, R., Winslow, D. N., & Vacanti, J. P. (1994). Preparation and characterization of poly(L-lactic acid) foams. *Polymer*, 35, 1068–1077.
- Mirzaei, E., Ai, J., Ebrahimi-Barough, S., Verdi, J., Ghanbari, H., & Faridi-Majidi, R. (2016). The differentiation of human endometrial stem cells into neuron-like cells on electrospun PAN-derived carbon nanofibers with random and aligned topographies. *Molecular Neurobiology*, 53, 4798–4808.
- Moon, S., Hasan, S. K., Song, Y. S., Xu, F., Keles, H. O., Manzur, F., ... Demirci, U. (2010). Layer by layer three-dimensional tissue epitaxy by cell-laden hydrogel droplets. *Tissue Engineering. Part C, Methods*, 16, 157–166.

- Mooney, E., Mackle, J. N., Blond, D. J., O'cearbaill, E., Shaw, G., Blau, W. J., ... Murphy, J. M. (2012). The electrical stimulation of carbon nanotubes to provide a cardiomimetic cue to MSCs. *Biomaterials*, 33, 6132–6139.
- Nguyen-Vu, T. D. B., Nguyen-Vu, T. D. B., Hua, C., Cassell, A. M., Andrews, R. J., Meyyappan, M., & Jun, L. (2007). Vertically aligned carbon nanofiber architecture as a multifunctional 3-D neural electrical interface. *IEEE Transactions on Biomedical Engineering*, 54, 1121–1128.
- Nimeskern, L., Avila, H. M., Sundberg, J., Gatenholm, P., Muller, R., & Stok, K. S. (2013). Mechanical evaluation of bacterial nanocellulose as an implant material for ear cartilage replacement. *Journal of the Mechanical Behavior of Biomedical Materials*, 22, 12–21.
- Noel, D., Gazit, D., Bouquet, C., Apparailly, F., Bony, C., Plence, P., ... Jorgensen, C. (2004). Short-term BMP-2 expression is sufficient for *in vivo* osteochondral differentiation of mesenchymal stem cells. *Stem Cells*, 22, 74–85.
- O'Brien, C., Holmes, B., Faucett, S., & Zhang, L. G. (2015). Three-dimensional printing of nanomaterial scaffolds for complex tissue regeneration. *Tissue Engineering Part B Reviews*, 21, 103–114. Available from <https://doi.org/10.1089/ten.TEB.2014.0168>.
- Ovsianikov, A., Gruene, M., Pflaum, M., Koch, L., Maiorana, F., Wilhelmi, M., ... Chichkov, B. (2010). Laser printing of cells into 3D scaffolds. *Biofabrication*, 2, 014104.
- Ovsianikov, A., Schlie, S., Ngezhahayo, A., Haverich, A., & Chichkov, B. N. (2007). Two-photon polymerization technique for microfabrication of CAD-designed 3D scaffolds from commercially available photosensitive materials. *Journal of Tissue Engineering and Regenerative Medicine*, 1, 443–449.
- Pan, L., Pei, X., He, R., Wan, Q., & Wang, J. (2012). Multiwall carbon nanotubes/polycaprolactone composites for bone tissue engineering application. *Colloids and Surfaces. B, Biointerfaces*, 93, 226–234.
- Parveen, S., Misra, R., & Sahoo, S. K. (2012). Nanoparticles: A boon to drug delivery, therapeutics, diagnostics and imaging. *Nanomedicine (London)*, 8, 147–166.
- Peng, Z., Zhao, T., Zhou, Y., Li, S., Li, J., & Leblanc, R. M. (2020). Bone tissue engineering via carbon-based nanomaterials. *Advanced Healthcare Materials*, 9, e1901495.
- Raimondi, M. T., Eaton, S. M., Lagana, M., Aprile, V., Nava, M. M., Cerullo, G., & Osellame, R. (2013). Three-dimensional structural niches engineered via two-photon laser polymerization promote stem cell homing. *Acta Biomaterialia*, 9, 4579–4584.
- Robbins, S., Lauryssen, C., & Songer, M. N. (2017). Use of nanocrystalline hydroxyapatite with autologous BMA and local bone in the lumbar spine: A retrospective CT analysis of posterolateral fusion results. *Clinical Spine Surgery*, 30, E192–E197.
- Sakamoto, A., Kiyokawa, K., Rikimaru, H., Watanabe, K., & Nishi, Y. (2012). An investigation of the fixation materials for cartilage frames in microtia. *Journal of Plastic, Reconstructive & Aesthetic Surgery: JPRAS*, 65, 584–589.
- Samadian, H., Mobasheri, H., Hasanpour, S., Ai, J., Azamie, M., & Faridi-Majidi, R. (2020). Electroconductive carbon nanofibers as the promising interfacial biomaterials for bone tissue engineering. *Journal of Molecular Liquids*, 298, 112021.
- Samavedi, S., Olsen Horton, C., Guelcher, S. A., Goldstein, A. S., & Whittington, A. R. (2011). Fabrication of a model continuously graded co-electrospun mesh for regeneration of the ligament-bone interface. *Acta Biomaterialia*, 7, 4131–4138.
- Sekiya, I., Colter, D. C., & Prockop, D. J. (2001). BMP-6 enhances chondrogenesis in a subpopulation of human marrow stromal cells. *Biochemical and Biophysical Research Communications*, 284, 411–418.
- Serrano, M. C., Nardecchia, S., Garcia-Rama, C., Ferrer, M. L., Collazos-Castro, J. E., Del Monte, F., & Gutierrez, M. C. (2014). Chondroitin sulphate-based 3D scaffolds containing MWCNTs for nervous tissue repair. *Biomaterials*, 35, 1543–1551.

- Shah, R. N., Shah, N. A., Del Rosario Lim, M. M., Hsieh, C., Nuber, G., & Stupp, S. I. (2010). Supramolecular design of self-assembling nanofibers for cartilage regeneration. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 3293–3298.
- Shim, J. H., Kim, J. Y., Park, M., Park, J., & Cho, D. W. (2011). Development of a hybrid scaffold with synthetic biomaterials and hydrogel using solid freeform fabrication technology. *Biofabrication*, 3, 034102.
- Shin, S. R., Jung, S. M., Zalabany, M., Kim, K., Zorlutuna, P., Kim, S. B., ... Khademhosseini, A. (2013). Carbon-nanotube-embedded hydrogel sheets for engineering cardiac constructs and bioactuators. *ACS Nano*, 7, 2369–2380.
- Shrestha, S., Shrestha, B. K., Kim, J. I., Ko, S. W., Park, C. H., & Kim, C. S. (2018). Electrodeless coating polypyrrole on chitosan grafted polyurethane with functionalized multiwall carbon nanotubes electrospun scaffold for nerve tissue engineering. *Carbon*, 136, 430–443.
- Silva, G. A., Czeisler, C., Niece, K. L., Beniash, E., Harrington, D. A., Kessler, J. A., & Stupp, S. I. (2004). Selective differentiation of neural progenitor cells by high-epitope density nanofibers. *Science (New York, NY)*, 303, 1352–1355.
- Smith, M. J., McClure, M. J., Sell, S. A., Barnes, C. P., Walpoth, B. H., Simpson, D. G., & Bowlin, G. L. (2008). Suture-reinforced electrospun polydioxanone-elastin small-diameter tubes for use in vascular tissue engineering: A feasibility study. *Acta Biomaterialia*, 4, 58–66.
- Song, S. J., Choi, J., Park, Y. D., Lee, J. J., Hong, S. Y., & Sun, K. (2010). A three-dimensional bioprinting system for use with a hydrogel-based biomaterial and printing parameter characterization. *Artificial Organs*, 34, 1044–1048.
- Sridharan, I., Kim, T., Strakova, Z., & Wang, R. (2013). Matrix-specified differentiation of human decidua parietalis placental stem cells. *Biochemical and Biophysical Research Communications*, 437, 489–495.
- Sun, L., Zhang, L., Hemraz, U. D., Fenniri, H., & Webster, T. J. (2012). Bioactive rosette nanotube-hydroxyapatite nanocomposites improve osteoblast functions. *Tissue Engineering. Part A*, 18, 1741–1750.
- Suri, S., Han, L. H., Zhang, W., Singh, A., Chen, S., & Schmidt, C. E. (2011). Solid freeform fabrication of designer scaffolds of hyaluronic acid for nerve tissue engineering. *Biomedical Microdevices*, 13, 983–993.
- Tarafder, S., Balla, V. K., Davies, N. M., Bandyopadhyay, A., & Bose, S. (2013). Microwave-sintered 3D printed tricalcium phosphate scaffolds for bone tissue engineering. *Journal of Tissue Engineering and Regenerative Medicine*, 7, 631–641.
- Tasoglu, S., & Demirci, U. (2013). Bioprinting for stem cell research. *Trends in Biotechnology*, 31, 10–19.
- Terrones, M. (2004). Carbon nanotubes: Synthesis and properties, electronic devices and other emerging applications. *International Materials Reviews*, 49, 325–377.
- Thomas, V., Zhang, X., & Vohra, Y. K. (2009). A biomimetic tubular scaffold with spatially designed nanofibers of protein/PDS bio-blends. *Biotechnology and Bioengineering*, 104, 1025–1033.
- Tokarev, A., Asheghali, D., Griffiths, I. M., Trotsenko, O., Gruz, A., Lin, X., ... Minko, S. (2015). Touch- and brush-spinning of nanofibers. *Advanced Materials*, 27, 6526–6532.
- Torgersen, J., Ovsianikov, A., Mironov, V., Pucher, N., Qin, X. H., Li, Z. Q., ... Stampfl, J. (2012). Photo-sensitive hydrogels for three-dimensional laser microfabrication in the presence of whole organisms. *Journal of Biomedical Optics*, 17, 105008. Available from <https://doi.org/10.1117/1.JBO.17.10.105008>.
- Tran, P. A., Zhang, L., & Webster, T. J. (2009). Carbon nanofibers and carbon nanotubes in regenerative medicine. *Advanced Drug Delivery Reviews*, 61, 1097–1114.
- Venclauskas, L., Grubinskas, I., Mocevicius, P., & Kiudelis, M. (2011). Reinforced tension line vs simple suture: A biomechanical study on cadavers. *Acta Chirurgica Belgica*, 111, 288–292.
- Wagner, D. E., Jones, A. D., Zhou, H., & Bhaduri, S. B. (2013). Cytocompatibility evaluation of microwave sintered biphasic calcium phosphate scaffolds synthesized using pH control. *Material Science & Engineering. C, Materials for Biological Applications*, 33, 1710–1719.

- Wang, M., Castro, N. J., Li, J., Keidar, M., & Zhang, L. G. (2012). Greater osteoblast and mesenchymal stem cell adhesion and proliferation on titanium with hydrothermally treated nanocrystalline hydroxyapatite/magnetically treated carbon nanotubes. *Journal of Nanoscience and Nanotechnology*, 12, 7692–7702.
- Wang, M., Cheng, X., Zhu, W., Holmes, B., Keidar, M., & Zhang, L. G. (2014). Design of biomimetic and bioactive cold plasma-modified nanostructured scaffolds for enhanced osteogenic differentiation of bone marrow-derived mesenchymal stem cells. *Tissue Engineering. Part A*, 20, 1060–1071.
- Wang, W., Li, B., Yang, J., Xin, L., Li, Y., Yin, H., ... Gao, C. (2010). The restoration of full-thickness cartilage defects with BMSCs and TGF-beta 1 loaded PLGA/fibrin gel constructs. *Biomaterials*, 31, 8964–8973.
- Wang, X., Wenk, E., Zhang, X., Meinel, L., Vunjak-Novakovic, G., & Kaplan, D. L. (2009). Growth factor gradients via microsphere delivery in biopolymer scaffolds for osteochondral tissue engineering. *Journal of Controlled Release*, 134, 81–90.
- Wang, X. L., Chen, Y. Y., & Wang, Y. Z. (2010). Synthesis of poly(p-dioxanone) catalyzed by Zn L-lactate under microwave irradiation and its application in ibuprofen delivery. *Journal of Biomaterials Science. Polymer Edition*, 21, 927–936.
- Warnke, P. H., Seitz, H., Warnke, F., Becker, S. T., Sivananthan, S., Sherry, E., ... Douglas, T. (2010). Ceramic scaffolds produced by computer-assisted 3D printing and sintering: Characterization and biocompatibility investigations. *Journal of Biomedical Materials Research. Part B, Applied Biomaterials*, 93, 212–217.
- Webster, T. J., Ergun, C., Doremus, R. H., Siegel, R. W., & Bizios, R. (2000). Specific proteins mediate enhanced osteoblast adhesion on nanophase ceramics. *Journal of Biomedical Materials Research*, 51, 475–483.
- Whang, K., Thomas, C. H., Healy, K. E., & Nuber, G. (1995). A novel method to fabricate bioabsorbable scaffolds. *Polymer*, 36, 837–842.
- Yang, B., Yin, J., Chen, Y., Pan, S., Yao, H., Gao, Y., & Shi, J. (2018). 2D-black-phosphorus-reinforced 3D-printed scaffolds: A stepwise countermeasure for osteosarcoma. *Advanced Materials*, 30. Available from <https://doi.org/10.1002/adma.201705611>.
- Yu, M. F., Lourie, O., Dyer, M. J., Moloni, K., Kelly, T. F., & Ruoff, R. S. (2000). Strength and breaking mechanism of multiwalled carbon nanotubes under tensile load. *Science (New York, NY)*, 287, 637–640.
- Zhang, C., Hu, Y., & Xu, J. (2005). The effect of bone-related growth factors on the proliferation and differentiation of marrow mesenchymal stem cells *in vitro*. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi = Zhongguo Xiu fu Chongjian Waikē Zazhi = Chinese Journal of Reparative and Reconstructive Surgery*, 19, 906–909.
- Zhang, L., Chen, Y., Rodriguez, J., Fenniri, H., & Webster, T. J. (2008). Biomimetic helical rosette nanotubes and nanocrystalline hydroxyapatite coatings on titanium for improving orthopedic implants. *International Journal of Nanomedicine*, 3, 323–333.
- Zhang, L., Ercan, B., & Webster, T. J. (2009). Carbon nanotubes and nanofibers for tissue engineering applications. In C. Liu (Ed.), *Carbon*. Research Signpost.
- Zhang, L., Hemraz, U. D., Fenniri, H., & Webster, T. J. (2010). Tuning cell adhesion on titanium with osteogenic rosette nanotubes. *Journal of Biomedical Materials Research. Part A*, 95, 550–563.
- Zhang, L., Hu, J., & Athanasiou, K. A. (2009). The role of tissue engineering in articular cartilage repair and regeneration. *Critical Reviews in Biomedical Engineering*, 37, 1–57.
- Zhang, L., Rakotondradany, F., Myles, A. J., Fenniri, H., & Webster, T. J. (2009). Arginine-glycine-aspartic acid modified rosette nanotube-hydrogel composites for bone tissue engineering. *Biomaterials*, 30, 1309–1320.

- Zhang, L., Ramsaywack, S., Fenniri, H., & Webster, T. J. (2008). Enhanced osteoblast adhesion on self-assembled nanostructured hydrogel scaffolds. *Tissue Engineering. Part A*, 14, 1353–1364.
- Zhang, L., Rodriguez, J., Raez, J., Myles, A. J., Fenniri, H., & Webster, T. J. (2009). Biologically inspired rosette nanotubes and nanocrystalline hydroxyapatite hydrogel nanocomposites as improved bone substitutes. *Nanotechnology*, 20, 175101.
- Zhang, L., Sirivisoot, S., Balasundaram, G., & Webster, T. J. (2008). Nanoengineering for bone tissue engineering. In A. Khademhosseini, J. Borenstein, M. Toner, & S. Takayama (Eds.), *Micro and nanoengineering of the cell microenvironment: Technologies and applications*. Artech House.
- Zhang, L. J., & Webster, T. J. (2009). Nanotechnology and nanomaterials: Promises for improved tissue regeneration. *Nano Today*, 4, 66–80.
- Zhang, S. (2003). Fabrication of novel biomaterials through molecular self-assembly. *Nature Biotechnology*, 21, 1171–1178.
- Zhang, Y., Wang, F., Tan, H., Chen, G., Guo, L., & Yang, L. (2012). Analysis of the mineral composition of the human calcified cartilage zone. *International Journal of Medical Sciences*, 9, 353–360.
- Zhou, X., Castro, N. J., Zhu, W., Cui, H., Aliabouzar, M., Sarkar, K., & Zhang, L. G. (2016). Improved human bone marrow mesenchymal stem cell osteogenesis in 3D bioprinted tissue scaffolds with low intensity pulsed ultrasound stimulation. *Scientific Reports*, 6, 32876.
- Zhou, X., Nowicki, M., Cui, H., Zhu, W., Fang, X., Miao, S., . . . Zhang, L. G. (2017). 3D bioprinted graphene oxide-incorporated matrix for promoting chondrogenic differentiation of human bone marrow mesenchymal stem cells. *Carbon*, 116, 615–624.
- Zhou, X., Tenaglio, S., Esworthy, T., Hann, S. Y., Cui, H., Webster, T. J., . . . Zhang, L. G. (2020). Three-dimensional printing biologically inspired DNA-based gradient scaffolds for cartilage tissue regeneration. *ACS Applied Materials & Interfaces*, 12, 33219–33228.
- Zhu, J., Dang, H. C., Wang, W. T., Wang, X. L., & Wang, Y. Z. (2011). Cellulose diacetate-g-poly(p-dioxanone) co-polymer: Synthesis, properties and microsphere preparation. *Journal of Biomaterials Science. Polymer Edition*, 22, 981–999.
- Zhu, W., Ma, X., Gou, M., Mei, D., Zhang, K., & Chen, S. (2016). 3D printing of functional biomaterials for tissue engineering. *Current Opinion in Biotechnology*, 40, 103–112.
- Zhu, W., Masood, F., O'Brien, J. R., & Zhang, L. G. (2015). Highly aligned nanocomposite scaffolds by electrospinning and electrospraying for neural tissue engineering. *Nanomedicine: Nanotechnology, Biology and Medicine*, 11(3), 693–704. Available from <https://doi.org/10.1016/j.nano.2014.12.001>.
- Zhu, W., O'Brien, C., O'Brien, J. R., & Zhang, L. G. (2014). 3D nano/microfabrication techniques and nanobiomaterials for neural tissue regeneration. *Nanomedicine: Nanotechnology, Biology, and Medicine*, 9, 859–875.
- Zhu, W., Ye, T., Lee, S.-J., Cui, H., Miao, S., Zhou, X., . . . Zhang, L. G. (2018). Enhanced neural stem cell functions in conductive annealed carbon nanofibrous scaffolds with electrical stimulation. *Nanomedicine: Nanotechnology, Biology and Medicine*, 14, 2485–2494.
- Zlotnick, H. M., Clark, A. T., Gullbrand, S. E., Carey, J. L., Cheng, X. M., & Mauck, R. L. (2020). Magneto-driven gradients of diamagnetic objects for engineering complex tissues. *Advanced Materials*, 32, 2005030.

Bioprinting of Biomimetic Tissue Models for Disease Modeling and Drug Screening

Min Tang, David Berry, Kathleen Miller, Xuanyi Ma and Shaochen Chen

Department of NanoEngineering, University of California, San Diego, CA, United States

2.1 INTRODUCTION

Three-dimensional (3D) bioprinting, an extension of 3D printing, is based on additive manufacturing technology and provides controlled fabrication of 3D structures in all X, Y, and Z directions (Irvine & Venkatraman, 2016; Murphy & Atala, 2014; Stanton, Samitier, & Sánchez, 2015). The complex structures to be formed can be designed using a computer-aided design (CAD) software or scanned from medical images including magnetic resonance imaging (MRI) or computed tomography (CT) scans (Collins, 2014; Giannopoulos et al., 2016). 3D bioprinting has emerged as a promising technology for fabricating complex tissue constructs with tailored biological components and mechanical properties (Murphy & Atala, 2014). By utilizing this transformative technology, bioinks, including hydrogels, cells, and growth factors, can be precisely positioned to create 3D *in vitro* culture environments (Jammalamadaka & Tappa, 2018; Skardal & Atala, 2015). In this way, native tissue architecture, cellular composition and vasculature can be recapitulated *in vitro* to create biomimetic tissue models, which can be used for studying disease mechanisms, screening drugs, and other clinical applications (Cui, Zhu, Holmes, & Zhang, 2016; Markstedt et al., 2015). With further involvement of induced pluripotent stem cell (iPSC)-derived cells, personalized tissue models in healthy and disease states can be fabricated to customize the drug screening and treatment process.

Here we review the development of *in vitro* tissue models using 3D bioprinting approaches. We first look at the state-of-the-art 3D bioprinting platforms, the commonly used cell source and biomaterial choices for building functional tissue models that can be used for personalized drug screening and disease modeling. Then we focus on 3D-bioprinted liver constructs, cardiac and skeletal muscle tissues, vascularized structures, and cancer models for their wide applications in medical research, drug discovery, toxicology, and other preclinical studies. Finally, we discuss the limitations of current technologies and the direction for future work.

2.2 CURRENT 3D BIOPRINTING APPROACHES TO BUILD BIOMIMETIC TISSUE MODELS

3D bioprinting has the advantage of reconstructing complex structures from CT or MRI images and producing accurate structures from predetermined digital designs such as CAD models. To build functional tissue models, the combination of 3D bioprinting technology with appropriate choice of cells and biomaterials is essential (Lee & Yeong, 2016; Murphy & Atala, 2014; Zhu et al., 2016). The fabrication capability of the 3D printer and the requirement on materials are highly dependent on the type of printer (Huang, Fu, Li, Dai, & Zhang, 2017; Ji & Guvendiren, 2017). The choice of cell source also leads to various application potentials of the tissue model (Leberfinger, Ravnic, Dhawan, & Ozbolat, 2017; Peng et al., 2017). In the following sections, we discuss these in more detail.

2.2.1 CURRENT 3D BIOPRINTING TECHNOLOGY

The primary types of 3D bioprinting technologies include inkjet-based, extrusion-based, and light-based printing. Each of the 3D-printing approaches has the capability to both print scaffolds for cell seeding and encapsulate cells directly within scaffolds to build tissue constructs. However, these platforms differ in various aspects including their printing mechanisms, resolution, time, and material choice. On the basis of recent publications in the past 3 years, we found that extrusion-based printing is the most used technique (Akkineni, Ahlfeld, Lode, & Gelinsky, 2016; AnilKumar et al., 2018; Apelgren et al., 2017; Bertlein et al., 2017; Bhuthalingam, Lim, Irvine, & Venkatraman, 2016; Byambaa et al., 2017; Chimene et al., 2018; Cochis et al., 2018; Colosi et al., 2016; Dai et al., 2017; Di Bella et al., 2018; Di Giuseppe et al., 2018; Diamantides et al., 2017; Dubbin, Tabet, & Heilshorn, 2017; Duchi et al., 2017; Ersumo, Witherel, & Spiller, 2016; Gao, He, Fu, Liu, & Ma, 2015; Gettler, Zakhari, Gandhi, & Williams, 2017; Gu, Tomaskovic-Crook, Wallace, & Crook, 2017; Ho & Hsu, 2018; Jia et al., 2016; Jin, Compaan, Bhattacharjee, & Huang, 2016; Jin, Liu, Chai, Compaan, & Huang, 2017; Kang et al., 2017; Kesti et al., 2015; Kim et al., 2016; Kim, Lee, Gao, & Cho, 2017; Koo & Kim, 2016; Lee, Yoo, Kang, & Cho, 2016; Liu, Heinrich, et al., 2017; Liu, Zhang, et al., 2017; Liu et al., 2018; McBeth et al., 2017; McElheny, Hayes, & Devireddy, 2017; Mistry et al., 2017; Müller, Öztürk, Arlov, Gatenholm, & Zenobi-Wong, 2017; O'Connell et al., 2016; Ouyang et al., 2015; Ouyang, Yao, Zhao, & Sun, 2016; Paxton et al., 2017; Reid et al., 2016; Ribeiro et al., 2017; Rocca, Fragasso, Liu, Heinrich, & Zhang, 2018; Rodriguez et al., 2018; Sakai, Ohi, Hotta, Kamei, & Taya, 2018; Schütz et al., 2017; Shafiee, McCune, Forgacs, & Kosztin, 2015; Shanjani, Pan, Elomaa, & Yang, 2015; Shao et al., 2015; Skardal et al., 2015; Tabriz, Hermida, Leslie, & Shu, 2015; Tan, Tan, Yeong, & Tor, 2016; Tijore, Behr, Irvine, Baisane, & Venkatraman, 2018; Wang, Highley, et al., 2018; Wu et al., 2016; Xu, Lee, Dai, & Hong, 2018; Yin, Yan, Wang, Fu, & Suo, 2018) followed by light-based (Bourget et al., 2016; Burks et al., 2016; Cadau et al., 2017; Dinh et al., 2017; Gi, Han, & Park, 2017; Jang et al., 2016; Kawecki et al., 2018; Keriquel et al., 2017; Koch et al., 2018; Miao et al., 2016; Morris, Nimbalkar, Younesi, McClellan, & Akkus, 2017; Sakai, Kamei, et al., 2018; Stichler, Böck, et al., 2017; Stichler, Jungst, et al., 2017; Vinson et al., 2017; Wang et al., 2015; Wang et al., 2017; Wang, Jin, et al., 2018; Xiong, Zhang, Chai, Huang, & Chrisey, 2015; Xiong, Zhang,

Chai, Chrisey, & Huang, 2017; Yang et al., 2015; Zhang, Xiong, Mei, Huang, & Chrisey, 2015; Zhang, Xu, Xiong, Chrisey, & Huang, 2017; Zhang, Chai, Xiong, Zhou, & Huang, 2017) and inkjet-based printing approaches (Benning et al., 2018; Christensen et al., 2015; Gao, Schilling, et al., 2015; Gao, Yonezawa, Hubbell, Dai, & Cui, 2015; Gao, Hubbell, Schilling, Dai, & Cui, 2017; Hart et al., 2016; Hendriks et al., 2015; Kim et al., 2017; Rimann, Bono, Annaheim, Bleisch, & Graf-Hausner, 2016; Sakai, Ueda, Gantumur, Taya, & Nakamura, 2018; Samson, Lee, & Song, 2018; Tse & Smith, 2018). Below we evaluate and compare these platforms more thoroughly.

2.2.1.1 Inkjet-Based Bioprinting

Inkjet-based bioprinting systems are modified from conventional desktop inkjet printers to dispense precise picoliter droplets of bioink (material solution or cell–material mixture) on printing stage (Figure 2.1A) (Hözl et al., 2016; Pereira & Bártolo, 2015). There are multiple approaches to inkjet printing, including thermal, piezoelectric, and electromagnetic (Cui, Boland, D’Lima, & Lotz, 2012). Among these types, the thermal approach is most commonly used because of the relatively high cell viability after printing, user-friendly design, and lower cost in general. During thermal inkjet printing, localized heating increases the temperature to 300°C for several microseconds and inflates an air bubble to push droplets out from the nozzle head (Cui et al., 2012). In the piezoelectric method, droplets are produced by the pulse pressure generated from a piezoelectric actuator (Li, Chen, Fan, & Zhou, 2016). The electromagnetic approach is based on electromagnetic

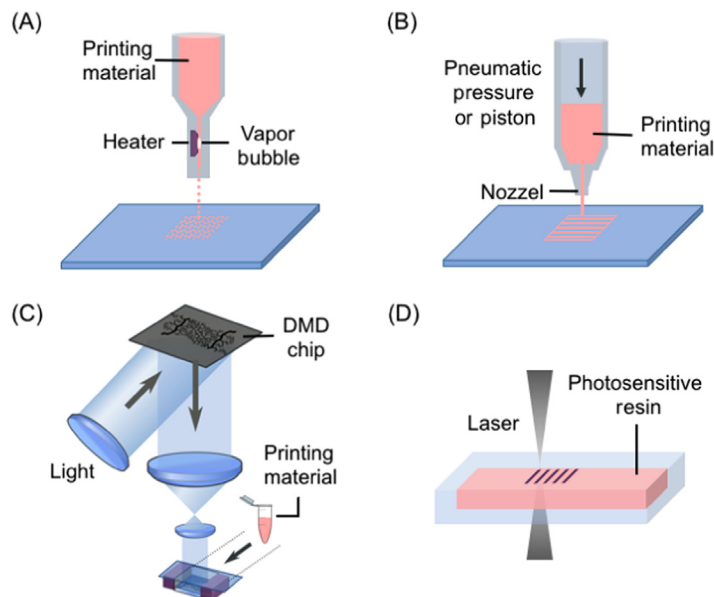


FIGURE 2.1

Schematic diagrams showing the printing approaches: (A) inkjet-based bioprinting systems, (B) extrusion-based bioprinting systems, (C) DLP-based bioprinting, and (D) TPP-based bioprinting platforms. *DLP*, digital light processing; *TPP*, two photon polymerization.

principles including Lorentz force and permanent magnet-based configurations (Andò & Marletta, 2016). Electromagnetic printing generates relatively larger droplets than thermal or piezoelectric approaches (Kollamaram, Hopkins, Glowacki, Croker, & Walker, 2018).

For current inkjet-based bioprinters, a printing speed in the range of hundreds of millimeters per second and a printing resolution as high as 20 μm has been reported (Kang et al., 2016; Zhu et al., 2016). Resolution of the printed constructs relies on the nozzle diameter as well as the properties of the bioink. Smaller diameter nozzle heads generally render higher printing resolution and also increase the potential for clogging; thus, the variety of materials that can be printed with inkjet-based method is limited. Generally, only materials with relatively low viscosity or water-based materials are suitable to minimize the chance of clogging. This requirement in turn limits the size and structural integrity of the constructs produced by this printing technology. While inkjet-based method is flexible and inexpensive, the limitations on materials, frequent nozzle clogging, slow printing speed due to point-by-point deposition, and potential damage to cells from shear or thermal stress are issues waiting to be resolved before the expansion of its applications to building more complex tissue models.

2.2.1.2 Extrusion-Based Bioprinting

Extrusion-based bioprinting systems deposit continuous filaments compared to the individual droplets of inkjet-based bioprinters (Figure 2.1B). This technology uses a set of automated motors to control the stage or the printer nozzle and a dispensing system to deposit bioink at a precise time and location that is digitally controlled by a computer. Multiple approaches can be used to drive the dispensing system, including pressure-based control, mechanical control, or solenoid control (Murphy & Atala, 2014). In this case, acellular or cell-laden bioinks can be printed onto a receiving substrate in a layer-by-layer fashion.

For microscale nozzle printing, a more versatile selection of bioinks is compatible with this technology. These include cell spheroid suspension, decellularized extracellular matrix (dECM) solutions, and hydrogels with a wider range of viscosity such as poly(ethylene glycol) (PEG)-based hydrogels, gelatin, hyaluronic acid (HA), and alginate (Axpe & Oyen, 2016; Graham et al., 2017; Jia et al., 2016; Ozbolat & Hospodiuk, 2016). Printing of more viscous hydrogels can provide a stronger mechanical support in the final structure. Notably, the flexibility of using more biocompatible inks during extrusion-based printing also make it more suitable for building a variety of tissue models. In addition to the wider choice of printing materials, extrusion-based printing is also advantageous in terms of printing and deposition speed as well as upscaling potential. However, extrusion-based bioprinting has the lowest reported printing speed among the three types of printing approaches, in the range of 10–50 $\mu\text{m/s}$ (Murphy & Atala, 2014; Zhu et al., 2016). Additionally, the resolution of the printed constructs is generally compromised to allow for 3D structures with a larger footprint. The reported minimal printed feature resolution can be 5 μm but it is generally over 100 μm (Murphy & Atala, 2014; Ozbolat & Hospodiuk, 2016; You, Eames, & Chen, 2017). Extrusion-based printing also suffers from shear induced cell death, which is similar to inkjet printing technology (Murphy & Atala, 2014; Ozbolat & Hospodiuk, 2016; You et al., 2017). Nevertheless, tissue models that lack microscale features such as bone, cartilage, and organoids can still be robustly built using extrusion-based bioprinting (Hung et al., 2016; Ozbolat & Hospodiuk, 2016; You et al., 2017). Furthermore, some biomaterials as well as tissues can be readily fabricated

by modeling with customized molds that are prepared by extrusion-based 3D-printing technology (Hu et al., 2016; Tao et al., 2017).

2.2.1.3 Light-Based Bioprinting

Light-based bioprinting methods have been developed and gained popularity for their high cell viability postprinting as well as superior printing speed and resolution. Different light-based printing platforms can address the challenges in building tissue models with various requirements. For example, two photon polymerization (TPP)-based printing provides superior feature resolution for building structure with nanoscale resolution, and digital light processing (DLP)-based printing is capable of rapid printing of complex architecture (Gao, Kupfer, et al., 2017; Ma et al., 2016).

2.2.1.3.1 TPP-Based Bioprinting

TPP-based bioprinting is a type of laser-based direct-writing technique developed from stereolithography, which generates structures by repeatedly and selectively photopolymerize photo-sensitive monomers with a rastering laser (Figure 2.1D) (Cumpston et al., 1999). The printing mechanism of TPP is based on the two-photon absorption phenomenon, where the probability of two-photon absorption by a molecule is associated with the square of light intensity (Zipfel, Williams, & Webb, 2003). This confines the dimension of the printing voxel to below $1\text{ }\mu\text{m}^3$ (Farsari & Chichkov, 2009). The feature resolution of TPP can achieve around 100 nm (Truby & Lewis, 2016), making it ideal for printing nanoscale features. The tradeoff of such high resolution is limitations in construct size and printing speed. The printing speed of TPP-based printers lies in the range of 200–1600 mm/s, as reported for laser assisted printers (Murphy & Atala, 2014; Zhu et al., 2016). A number of polymers have been successfully printed with this method, including type I collagen (Bell, Kofron, & Nistor, 2015), bovine serum albumin (Engelhardt et al., 2011), laminin (Su et al., 2012), streptavidin (Wylie et al., 2011), and PEG-based hydrogels (Zhang, Soman, Meggs, Qu, & Chen, 2013).

2.2.1.3.2 DLP-Based Bioprinting

DLP-based bioprinting systems incorporate light sources, digital micromirror device (DMD) chips, motorized stages, a holder for bioinks, an optional motorized printing head, and a computer control system that coordinates all components (Figure 2.1C) (Gou et al., 2014; Hribar, Soman, Warner, Chung, & Chen, 2014). The DMD chip consists of millions of micromirrors that allow for precise light projection patterning by turning individual micromirror to the “on” or the “off” state independently throughout the printing process. Light is projected onto the prepolymer bioink only when the micromirror is in its arbitrary “on” state (Zhu et al., 2016). The resolution of DLP-based printers is usually at the microscale level, depending on the focal size of the light beam from each of the micromirrors. With DLP-based bioprinting, an entire plane of pattern is projected onto the prepolymer solution at a time, and the stage moves while the printing patterns continuously refresh. Absence of the artificial interfaces between dots as in inkjet-based printing or between lines as in extrusion-based printing provides better mechanical integrity of the printed structure. The projection approach also results in a printing speed much faster than that of other conventional approaches, usually at a few cubic millimeters per second (Zhu et al., 2016).

DLP-based approaches with flexible pattern input, rapid printing speed, and high printing resolution allow researchers to build complex structures with high precision, including microwells

(Hribar et al., 2015), microfluidic mixing chambers (Liu, Hwang, Wang, Whang, & Chen, 2016), fractal geometries (Warner, Soman, Zhu, Tom, & Chen, 2016), and constructs with tunable Poisson ratios (Lee, Soman, Park, Chen, & Cho, 2016; Soman, Fozdar, et al., 2012). Two recently developed volumetric printing approaches including computed axial lithography and xolography enable one-step 3D construct fabrication with a high volume generation rate and the potential to fabricate overhanging features or free-floating objects (Kelly et al., 2019; Regehy et al., 2020). With the capability of modulating scaffold mechanical property, DLP-based bioprinting can also be used to print tissue models and disease models (Cha et al., 2014; Grogan et al., 2013; Ma et al., 2016; Soman, Tobe, et al., 2012; Zhu et al., 2017), including but not limited to vasculature network (Zhu et al., 2017), aligned muscle scaffolds (Liu et al., 2020; Ma et al., 2019), and liver microarchitecture (Ma et al., 2016). Materials that are compatible with this printing technique include various photopolymerizable polymers, such as gelatin methacrylate (GelMA), poly(ethylene glycol) diacrylate (PEGDA), glycidyl methacrylate HA (GMHA), thiolated heparin (Hep-SH), and poly(glycerol sebacate) acrylate (PGSA) (Wang et al., 2019, 2020; Zhong et al., 2021).

Relatively limited selection of bioinks compared to other two types of printing approaches is a primary limitation of light-based bioprinting, which requires further efforts in material engineering. In addition, light-based printing platforms mostly use a printing reservoir or chamber to contain bioinks that often leads to wasted unpolymerized bioink consisting of cells and biomaterials that are costly and precious (Murphy & Atala, 2014). By addressing these current limitations, light-based printing will broaden its application in the biomedical field.

2.2.2 CELL SOURCE AND PREPARATION

Most tissues are not acellular; therefore, incorporation of cells is essential to create functional tissue constructs. To build 3D-printed *in vitro* tissue models, there are two ways of cell incorporation: cell seeding onto already printed scaffolds and cell encapsulation during the printing process. For the seeding method, cells can be seeded directly or mixed together with a carrier matrix like collagen. The latter approach has been more popular for cell types that are sensitive, less proliferative, and dependent on cell matrix interactions (Hastings et al., 2015; Murphy & Atala, 2014; Steele, MacArthur, & Woo, 2017). In the case of cell encapsulation, a cell suspension solution is premixed with the biomaterial solution and allowed to solidify through various methods depending on the printing approach. Overall, the choice of cellularization approach depends on a variety of factors such as the intended purpose of study, printing method employed, and the cell type used.

In general, there are mainly three sources of cells commonly used for building 3D-printed tissue models: primary cells, cell lines, and stem cell-derived cells. Primary cells are cells directed isolated from human or animal tissues. If these cells are properly harvested from the targeted tissue at the desired health stage, they are great candidates to recapitulate the specific tissue functions at the specific point or stage (Caddeo, Boffito, & Sartori, 2017). However, the availability of human primary cells is low and sometimes very rare like in the case of primary cardiomyocytes. In addition, there are always batch-to-batch or donor-to-donor variations. The tissue constructs that use primary cells are also not patient specific, making it less preferable for personalized platforms. Cell lines are cells that can be subcultured repeatedly *in vitro* and have acquired homogenous genotypic and phenotypic characteristics. Cell lines are cheaper and easily accessible, and likely have standard culture procedure. For these reasons, cell lines are good choices when used as the starting or testing cells for

building new tissue models. They are also widely used in cancer models when focusing on specific cancer cell behavior. However, these cells are mostly modified so their structures and functional performance may differ from the targeted cells. Like primary cells, they cannot be applied to personalized platforms. Stem cell–derived cell type is the third kind of cells commonly used in 3D-printed tissue constructs. When primary cells are less available and cell lines are not ideal, stem cell–derived cells are often good choices to consider. These include mesenchymal stem cells (MSC), embryonic stem cells (ESC), and iPSC-derived cells. In particular, human iPSC-derived cells are gaining increasing popularity for their potential to recapitulate individual differences. They are widely applied in many kinds of *in vitro* tissue models. These cells, however, still have limitations in that they are often not functionally mature enough and also there can be inconsistency between differentiation batches (Li, Zhu, & Young, 2017; Medvedev, Shevchenko, & Zakian, 2010).

Recently coculture platforms are gaining increasing attention due to the better support they provide on cell survival and tissue function (Gao, Kupfer, et al., 2017; Ma et al., 2016; Xu et al., 2011; Zhu et al., 2017). Therefore including multiple types of cells in the printing process is becoming a more common strategy. Most of the coculture platforms are printed by extrusion- and light-based bioprinters (Gao, Kupfer, et al., 2017; Ma et al., 2016; Xu et al., 2011; Zhu et al., 2017). Due to the addition of multiple cell types, these platforms make it possible to study interactions between different cell types and provide paracrine support from nonparenchymal cell types. Such benefits are particularly significant when studying cancer behaviors and modeling organs whose function rely on the contributions from multiple cell types.

Regardless of the type of cell source chosen, there can always be high variations between cells used for different batches of 3D printing. Such variations come from a variety of sources including but not limited to user handling techniques, culture medium and chemicals (Yao & Asayama, 2017), variations in culture environment (Yao & Asayama, 2017), aging and mutations of cells (Kinarivala, Shah, Abbruscato, & Trippier, 2017; Liu et al., 2014; Morrison et al., 2016), and differentiation inconsistency (Morrison et al., 2016). Therefore implementing certain assays or methods to characterize cells before printing is essential to achieve consistency and reproducibility between experiments (Carmen, Burger, McCaman, & Rowley, 2012; Wang, Xu, Luo, Zhou, & Si, 2018). The specific assay and methods chosen are usually highly cell type dependent, but general characterizations on cell viability, purity, and phenotype can be applied to all cell types (Fang & Eglen, 2017; Ma et al., 2016; Wang, Xu, et al., 2018). Such characterizations can also be used following printing to study the impacts on cells due to the cell preparation technique, printing process, and 3D culture method.

2.2.3 BIOMATERIAL CHOICE

To design tissue scaffolds with the desired physical and chemical properties in 3D bioprinting, proper biomaterial selection is an important consideration. More specifically, biomaterials can be divided into two main categories: naturally derived [e.g., collagen (Inzana et al., 2014), gelatin (Gomes, Leonor, Mano, Reis, & Kaplan, 2012), fibrin (Gomes et al., 2012), HA (Gomes et al., 2012), silk proteins (Gomes et al., 2012), chitosan (Almeida et al., 2014), alginate (Axpe & Oyen, 2016), and dECM (Pati et al., 2014)] and synthetic [e.g., poly(lactic acid) (PLA) (Almeida et al., 2014), poly(lactic-glycolic) acid (PLGA) (Kim, Choi, Kim, Chan Choi, & Cho, 2010), PEGDA (Liu et al., 2016), etc.]. Naturally derived materials are attractive because the complexity of their biophysical and biochemical constituents closely recapitulates the native ECM. In turn, these

intrinsic properties have been demonstrated to strongly support cell adhesion, proliferation, differentiation, migration, and biocompatibility (Keane & Badylak, 2014). However, natural biomaterials are often mechanically weak with higher potential for variation between batches. Synthetic materials are highly defined and can be easily reproduced to control for a wide range of properties including degradation rate, cell adhesive moieties, mechanical strength, and structure (Keane & Badylak, 2014). For instance, synthetic polymer backbones can be decorated with cell recognition peptide sequences such as RGD and YIGSR to improve cell adhesion onto the substrates (Zhu, 2010). This flexibility enables the user to adopt a bottom-up approach to engineer a microenvironment mimicking the chemical and physical elements of the ECM found *in vivo*. Despite these advantages, it remains difficult to fully recapitulate components of the native tissue ECM artificially and the potential for poor tissue integration as well as the production of cytotoxic degradable byproducts may pose concerns with respect to long-term biocompatibility (Lu et al., 2000).

To circumvent these challenges, composite hydrogels incorporating both natural and synthetic biomaterials have been employed by combining the advantages of both to better emulate the characteristics of natural tissues. More specifically, synthetic materials can be used to impart mechanical strength, while naturally derived materials contribute ECM components into the hydrogel matrix to improve cell viability and functionality. For example, Hutson et al. developed PEG-GelMA hydrogels that can be copolymerized to exhibit tunable stiffness and degradation profiles with improved fibroblast binding and viability compared to PEG only hydrogels (Hutson et al., 2011). Interpenetrating hydrogel networks composed of polyvinyl alcohol (PVA)-gelatin and a PEG porogen have also been used by Miao et al. to vary modulus (i.e., 10–100 kPa) by modifying the concentration and molecular weight of PVA as well as the gelatin content for cartilage regeneration (Miao, Miller, McKenzie, & Oldinski, 2015). In alternative approaches, the ability to deposit multimaterial in 3D printing has also been employed by depositing synthetic materials such as polydimethylsiloxane and poly(caprolactone) (PCL) to fabricate a supportive framework into which natural materials including collagen, gelatin, fibrin, and dECM may be subsequently placed in between (Kolesky, Homan, Skylar-Scott, & Lewis, 2016; Lee et al., 2017; Pati et al., 2014). Furthermore, nanoparticles could be incorporated into the hydrogels to create functional structures. For instance, Gou et al. printed a 3D liver-mimetic hydrogel with nanoparticles that can effectively cleanse the blood (Gou et al., 2014).

As we move toward the production of biomimetic tissues, there is an increasing need to develop novel biomaterials that possess complex biophysical and biochemical cues to promote tissue-specific function and maturation. While most naturally derived bioinks utilize gelatin, collagen, and HA, these materials only represent single components of the ECM and lack other important constituents such as growth factors, proteoglycans, glycosaminoglycans, laminin, fibronectin, and elastin (Crapo, Gilbert, & Badylak, 2011). As a result, the use of dECM derived from tissues and organs has gained interest for applications in tissue engineering and regenerative medicine. The process of decellularization aims to remove all cellular components using a combination of mechanical, chemical, and enzymatic treatments to yield a collagenous matrix material while retaining constituents of the native ECM. Studies have also demonstrated that ECM derived from different tissues are compositionally distinct and cells respond to these matrices in a tissue-specific manner that is important in maintaining phenotype and functionality (Beachley et al., 2015; Crapo et al., 2011; Russo, Yu, Belliveau, Hamilton, & Flynn, 2014). With regard to 3D bioprinting, seminal work by Pati et al. showed the development of decellularized tissue bioinks from pepsin solubilized cardiac,

adipose, and cartilage tissues to fabricate cell-laden constructs with the use of a nozzle-based printer. These dECM-printed constructs enhanced functionality of encapsulated rat myoblasts, human adipose-derived stem cells, and human inferior turbinate-tissue–derived MSC for each of the cardiac, adipose, and cartilage printed constructs, respectively, in comparison to collagen bioink controls (Pati et al., 2014). This study demonstrates the versatility of dECM in 3D bioprinting to fabricate tissue constructs to better recapitulate the microenvironment of *in vivo* tissues and organs.

In the context of 3D bioprinting, a range of mechanisms have been employed to print both naturally derived and synthetic hydrogel precursors. Regardless of the bioink selected, the biomaterials must be able to quickly form a hydrogel network during the printing process either through chemical or physical crosslinking mechanisms. For example, in light-based 3D bioprinting systems crosslinking is achieved through free-radical polymerization of photopolymerizable bioinks (Kolesky et al., 2014), whereas in nozzle-based printing modalities other methods including thermal gelation (Laronda et al., 2017), ionic crosslinking (Axpe & Oyen, 2016), and via pH sensitivity (Yoon, Lee, Yim, Kim, & Chun, 2016) have been used. In addition to the crosslinking mechanism employed, the properties of the bioink must be considered and carefully selected for optimizing printing parameters in different 3D-printing systems. In extrusion-based 3D printing, a major consideration for users is solution viscosity. Lower viscosity materials may implement aforementioned crosslinking methods near the extruder tip, whereas higher viscosities allow the printing of self-supporting structures that maintain their shape until crosslinking occurs. In this case, adjustments must be made to the nozzle gauge and printing speed to accommodate material viscosity or using materials with shear thinning properties (Highley, Rodell, & Burdick, 2015). Other factors including platform temperature and humidity along with shear stress through the extruder are also important considerations, especially during the direct printing of cells with extrusion-based methods (Smith, Christian, Warren, & Williams, 2007). In light-based 3D bioprinting, both fluid and viscous materials are compatible, thus opening users to materials with a larger range of mechanical properties. However, these printing systems are limited to photocurable bioinks, which require synthetic and natural biomaterials to be functionalized with photo-crosslinkable groups such as PEGDA, GelMA, GMHA, Hep-SH, and PGSA. In addition, the opacity of the chosen biomaterial is also an important consideration because this will impact the light penetration depth and subsequently affect the resolution and quality of the final structure (Zhu et al., 2015).

The decision on the choice of biomaterials depends on a variety of factors, including but not limited to the type of printing approach, tissue of interest, mechanical properties, and the biological process to model. To build *in vitro* tissue models for disease modeling and personalized drug screening, future research is needed to develop materials with high tunability on the mechanical, chemical, and biological properties to recapitulate the protein composition as well as the native tissue environment at the targeted health stage.

2.3 DRUG SCREENING AND DISEASE MODELING APPLICATIONS IN VARIOUS ORGANS

In the following sections, the studies utilizing 3D bioprinting technologies to build liver, cardiac and skeletal muscle, and cancer models are discussed. More specifically, the use of different 3D

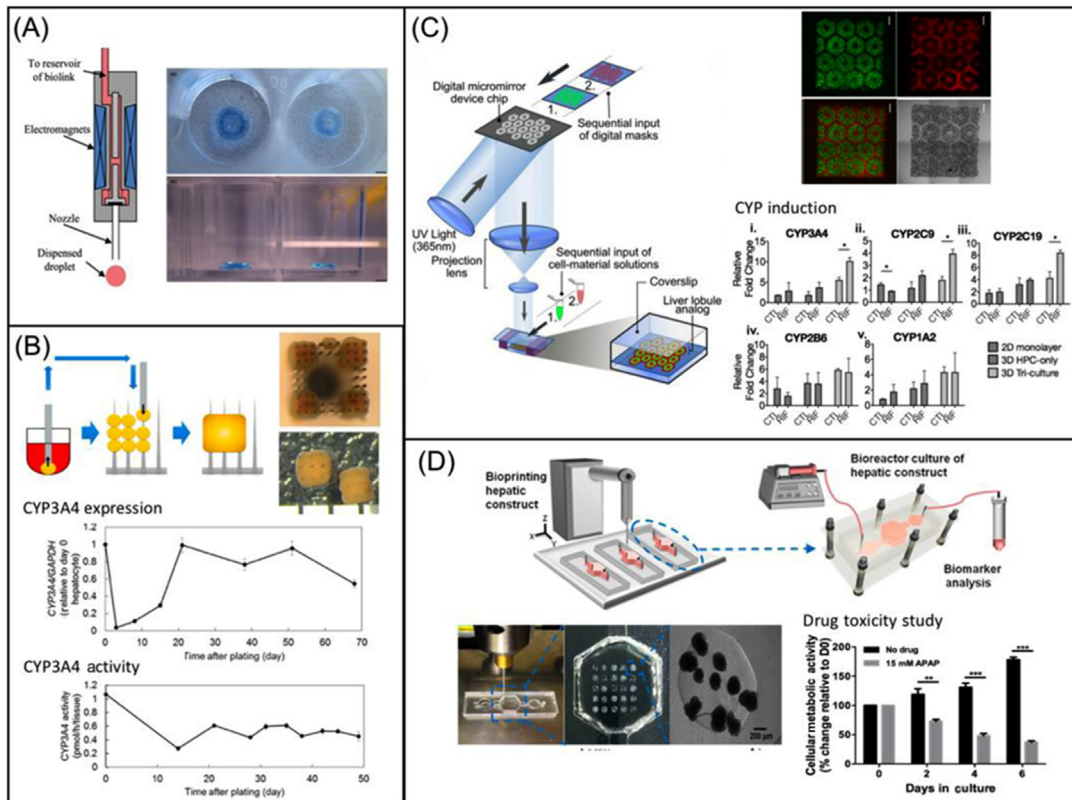
bioprinting approaches as well as the performance of current 3D-printed tissue constructs in terms of their tissue-specific functions, drug metabolizing potentials, and drug–dose responses are reviewed.

2.3.1 LIVER MODELS

Liver associated diseases are major contributors of morbidity and mortality in the United States (Scaglione et al., 2015). These abnormalities can lead to the formation of excessive fibrous tissue, result in the reduction of both liver-specific and systematic functions, and transition to nonreversible end-stage liver failure that can only rely on liver transplantation (Bataller & Brenner, 2005). In addition, as the liver serves a vital role in xenobiotic metabolism and detoxification, the investigation of hepatotoxicity is an essential component of any preclinical drug study (Kaplowitz, 2005). Conventional animal models are often costly and unreliable in translation to human studies due to variations in hepatocellular functions of different species (Hewitt et al., 2007; Khetani & Bhatia, 2008; Tsang et al., 2007). Moreover, both liver disease progression and drug response vary between individuals. Failure in hepatotoxicity prediction can lead to postmarket withdrawal of a drug. Therefore effective *in vitro* human liver models that based on personalized cell type are considered as a highly promising approach to better understand the disease mechanism, serve as a drug screening platform, and potentially treat disease in a regenerative medicine approach.

Over the past decades, liver tissue engineering has made significant progress toward the establishment of *in vitro* liver models for both fundamental pathophysiological studies and drug screening (Bhatia, Underhill, Zaret, & Fox, 2014; Guguen-Guillouzo, Corlu, & Guillouzo, 2010; Sivaraman et al., 2005; Yoon No, Lee, Lee, & Lee, 2015). The sources of cells used for these *in vitro* liver models include primary hepatocytes, hepatic cell lines isolated from tumors or liver slices, and stem cell–derived hepatic cells (Hewitt et al., 2007; Khetani & Bhatia, 2008; Sivaraman et al., 2005). Monolayer culture, organoid culture, and coculture platforms have been established using culture plates (Du et al., 2008; Takayama et al., 2013), commercially available wells (Gaskell et al., 2016), microfluidic perfusable chips (Domansky et al., 2010; Toh et al., 2009), dielectrophoresis micropatterning (Ho et al., 2013), and physical mask-based additive photopatterning methods (Khetani & Bhatia, 2008). However, the liver-specific functions of hepatocytes cultured in such platforms declined over weeks of *in vitro* culture (Berger, Ware, Davidson, Allsup, & Khetani, 2014; Du et al., 2008; Kim, Ohashi, Utoh, Kano, & Okano, 2012; Sivaraman et al., 2005; Takayama et al., 2013). Therefore liver constructs that better mimic native environment and help maintain *in vitro* liver functions is in great demand. 3D bioprinting technology, with its potential to pattern cells and biomaterials in a precise manner, provides a great tool to achieve novel and biomimetic *in vitro* liver models with increasing structural complexity.

Different 3D bioprinting approaches have been utilized to create liver tissue constructs. Faulkner-Jones et al. reported the use of inkjet-based bioprinter to encapsulate human iPSC and ESC-derived hepatocyte-like cells (HLCs) in alginate hydrogels to create 3D ring structures (Figure 2.2A) (Faulkner-Jones et al., 2015). Alginate was chosen based on its good biocompatibility, low immunogenicity, low toxicity, and hydrophilic nature (Faulkner-Jones et al., 2015). The cell-laden alginate droplets were exposed to calcium chloride solution followed by barium chloride before incubating in culture medium (Faulkner-Jones et al., 2015). The viability and albumin secreting function of HLCs were well maintained following this valve-based bioprinting. Kang

**FIGURE 2.2**

3D bioprinting of liver tissue models: (A) schematic diagram showing the inkjet-based printing setup (left) and bright field image showing the printed construct stained in blue (right). (B) Schematic diagram showing the process of building the construct from spheroids, with the top and side views of the construct on the top right. Plots showing expression (middle) and activity (bottom) of CYP3A4 over time. (C) Schematic diagram showing the DLP-based bioprinting system, with the fluorescence and bright field images of 3D-printed liver construct on the top right. Bar charts showing CYP enzyme induction on lower right. Scale bars are 500 μm . (D) Schematic diagram showing the direct 3D printing within microfluidic chip, with images showing the microfluidic setup and printed structure on lower left. Bar chart showing drug toxicity study on bottom right.

(A) Reprinted from [Faulkner-Jones et al. \(2015\)](#); (B) Reprinted from [Kizawa et al. \(2017\)](#); (C) Reprinted from [Ma et al. \(2016\)](#); (D) Reprinted from [Bhise et al. \(2016\)](#).

et al. used extrusion-based bioprinting to generate a 3D hepatic structure ([Kang et al., 2018](#)). Here, a five-layer alginate scaffold containing mouse induced HLCs, each measuring 25 mm $\times$ 25 mm, was constructed. During *in vitro* culture, the expression of albumin, ASGR1, and HNF4a gradually increased ([Kang et al., 2018](#)). The construct was also transplanted *in vivo* with increased proliferation and higher albumin expression observed ([Kang et al., 2018](#)). This work demonstrated the use of 3D-bioprinted liver scaffold as an effective option for liver therapy. Kizawa et al. also

demonstrated a scaffold-free 3D bioprinting technology to build liver tissue that could maintain stable bile acid secretion as well as drug, glucose, and lipid metabolism for weeks (Figure 2.2B) (Kizawa, Nagao, Shimamura, Zhang, & Torii, 2017). This was achieved by connecting spheroids of human primary hepatocytes using a 3D bioprinter. Their work provided insight on the long-term culture of 3D-bioprinted liver constructs *in vitro*. In particular, the group studied the expression and activity of CYP3A4 enzymes and showed that both were maintained for a period of 2 months. To mimic the complex microarchitecture of liver, Ma et al. reported the use of DLP-based bioprinting technology to build biomimetic liver tissue at microscale resolution (Figure 2.2C) (Ma et al., 2016). The 3D-bioprinted liver construct consisted of a hexagonal array of human iPSC-derived hepatic cells with supporting cells (Figure 2.2C). Hepatic cells cultured in this 3D-bioprinted tri-culture model demonstrated better liver-specific function and drug metabolism potential following CYP induction than those cultured in conventional two-dimensional (2D) monolayer and 3D single culture platforms (Ma et al., 2016). Incorporation of dECM in bioprinted liver models provided conducive environment for the maturation of iPSCs-derived hepatocytes and improved liver functionality (Yu et al., 2019). Liver dECM provides essential biochemical and structural cues to the hepatic cells to maintain their native phenotypes. Ma et al. also reported using liver dECM and DLP-based bioprinting to construct hepatocellular carcinoma models (Ma et al., 2018). Scaffolds with stiffness covering normal tissue to cirrhotic liver were created using photopolymerizable dECM to investigate liver cancer progression at different stages of liver cirrhosis. Directly printing into a microfluidic chamber to build liver-on-a-chip platform has also been demonstrated by Bhise et al. (2016) (Figure 2.2D). Droplets of HepG2 spheroid–GelMA mixture were printed on a glass slide within the cell culture chamber of a bioreactor, followed by immediate UV crosslinking (Bhise et al., 2016). The engineered hepatic construct remained functional during the 30-day culture period and showed a drug response similar to published data (Bhise et al., 2016).

The applications of 3D-printing technology to build *in vitro* liver models as shown in the above examples have demonstrated great benefits in providing long-term culture with well-maintained liver-specific functions and drug metabolism potential. Nevertheless, maintaining functional liver cell functions for longer than 30 days and achieving drug response profiles comparable to native liver still remain as great challenges in the field.

2.3.2 CARDIAC AND SKELETAL MUSCLE MODELS

2.3.2.1 Cardiac Muscle

Cardiovascular diseases are the foremost cause of death in the United States (Mozaffarian et al., 2015). Roughly 1 billion US dollars are spent researching and developing a new drug, only to fail clinical trials at rates as high as 80% for cardiovascular drugs (Ciociola, Cohen, Kulkarni, & FDA-Related Matters Committee of the American College of Gastroenterology, 2014). Furthermore, some drugs may disproportionately benefit or harm certain genotypes, ethnicities, sexes, and ages, while clinical trials are not necessarily representative of possible users of the drug (Ribas et al., 2016). Cardiotoxicity is often evaluated in cell cultures missing the native 3D extracellular micro-environment, inducing nonphysiological alignment and therefore compromising intercellular communication, profoundly confounding results. Consequently, cardiotoxicity is the primary reason for the retraction of pharmaceuticals from the market (Mozaffarian et al., 2015). Hence there is

significant need for predictive preclinical models, which are currently reliant on animal models lacking translational relevance to humans, as well as a biomimetic platform for personalized drug screening.

As most users of pharmaceuticals are adult humans, primary human adult cardiomyocytes are an ideal cell source; however, being terminally differentiated, they cannot expand in culture and acquiring more of these cells would necessitate the routine collection of biopsies of heart tissue from patients. Alternative cell sources of interest are human ESC-derived cardiomyocytes and human iPSC-derived cardiomyocytes (iPSC-CMs) (Lundy, Gantz, Pagan, Filice, & Laflamme, 2014). With the potential to represent individual differences, human iPSC-CMs are more preferred in studying disease mechanisms and for screening potential therapeutic drugs. Biomaterials are widely used as the scaffold for developing cardiac tissues, and so must mimic biochemical and mechanical properties of the native ECM. Commonly employed natural biomaterials for cardiac tissue engineering include ECM proteins such as fibrin (Ye, Sullivan, & Black, 2011), collagen (Legant et al., 2009), gelatin (McCain, Agarwal, Nesmith, Nesmith, & Parker, 2014), and decellularized cardiac matrix (Pati et al., 2014; Singelyn et al., 2009). Synthetic options include polyacrylamide hydrogels, which allow for independent control of both mechanical and biochemical properties (Gribova, Crouzier, & Picart, 2011). These versatile hydrogels have elastic moduli that can be tuned to mimic the elasticity of both healthy (Ribeiro et al., 2015) and infarcted myocardium (Engler et al., 2008) (10–15 and >50 kPa, respectively) and can attach to the user's choice of ECM proteins (Chaudhuri, Ramachandran, Moharil, Harumalani, & Jaiswal, 2017).

Most work toward developing these disease modeling and drug discovery/screening platforms has focused on recreating microtissues of the left ventricular myocardium, the site of most cardiac pathologies and the primary pumping chamber of the heart. These microtissues are generally created by seeding cells atop or encapsulating cells within a scaffold that mimics the ECM by supporting and directing tissue growth (Hirt, Hansen, & Eschenhagen, 2014). Cardiomyocytes in heart interact with microscale features within a 3D multilayered construct. 3D bioprinting is thus a promising fabrication tool as it offers unprecedented control of 3D architecture, able to create arbitrarily complex geometries in fine detail with high precision and accuracy, and is superior to traditional methods of creating 3D scaffolds (e.g., electrospinning, freeze-drying, gas-foaming, and particle or porogen leaching) which only allow for control of bulk properties (Murphy & Atala, 2014; Zhu et al., 2016). Precisely patterned microtopological and biochemical cues can promote the growth of confluent, aligned, as well as structurally and electrically anisotropic tissue to match the structure and function of native myocardium (Rao et al., 2013; Salick et al., 2014). 3D bioprinting also allows for the direct patterning of cells (Murphy & Atala, 2014), avoiding the inevitable and potentially confounding effect of cell aggregation due to relatively uncontrolled cell distribution in the traditional approach of seeding cells into a prefabricated scaffold. Furthermore, 3D printing can accommodate an ever-growing library of biomaterials with tunable mechanical and biochemical properties and allows for significantly simplified fabrication, rapid iteration, and increased dimensionality compared to traditional patterning techniques such as microcontact printing/micromolding.

Cardiac tissues have been bioprinted using iPSC-CMs and primary cardiac cells through different printing methods. iPSC-CMs and endothelial cells have been encapsulated through extrusion-based printing in an alginate-PEG-fibrinogen hydrogel to produce a prevascularized cardiac patch (Maiullari et al., 2018). Additionally, Lind et al. designed microarchitectures that guide the self-

assembly of laminar rat-derived cardiac tissues, embedding noninvasive contractile stress sensors (Figure 2.3A) (Lind et al., 2016). The system was fully fabricated via direct ink writing multimaterial 3D printing of six functional bioinks based on highly conductance, piezoresistive, and biocompatible soft materials. This microphysiological device acquires data that is delivered electronically as opposed to optically, reducing labor, eliminating the need for dedicated microscopy setups, and

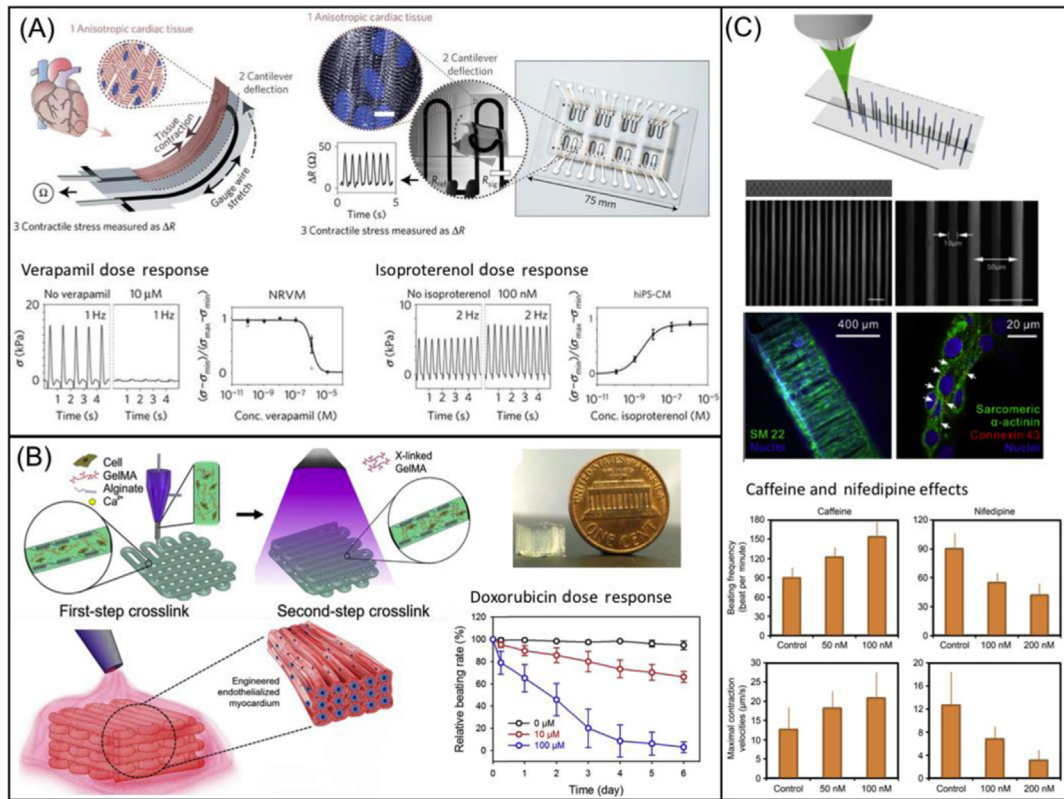


FIGURE 2.3

3D bioprinting of cardiac tissue models: (A) schematic diagram showing the design of a multimaterial, patterned, piezoresistive stress sensor that aligns cardiomyocytes in a thick tissue and sense cardiac force output by changes in resistivity during contraction. Scale bar are 10 μ m. Plots showing verapamil and isoproterenol dose response are on the bottom. (B) Schematic diagram showing the extrusion-based 3D-printing system that generate multimaterial prints of cardiomyocytes and endothelial cells in naturally based alginate and GelMA scaffold. The image of printed construct is shown on the top right and the doxorubicin dose response is shown on the lower right. (C) Schematic diagram showing TPP-based printing of micron-scale filaments, which was seeded with healthy and Long-QT iPSC-CMs. Fluorescence images in the middle showing the construct in bulk and cell alignment in various conditions. Bar charts on the bottom showing effects of caffeine and nifedipine on beating frequency and maximal contraction.

(A) Reprinted from Lind et al. (2016); (B) Reprinted from Zhang et al. (2016); (C) Reprinted from Ma et al. (2014).

allowing measurements to be taken in an incubator. The engineered microtissue exhibited inotropic responses to L-type calcium channel blocker, verapamil, and β -adrenergic agonist isoproterenol, comparable to data from engineered 3D neonatal rat ventricular myocardial tissues and isolated postnatal whole rat hearts, demonstrating the model's potential as a drug screening platform (Lind et al., 2016). Zhang et al. also reported the use of a composite alginate/GelMA bioink mixed with endothelial cells to directly print a hydrogel scaffold through a combination of extrusion and photocuring process (Figure 2.3B) (Zhang et al., 2016). The endothelial cells gradually migrated toward the microfiber peripheries, forming a controlled anisotropic, confluent layer of endothelium, which was then seeded with rat-derived cardiomyocytes (Zhang et al., 2016). An aligned, spontaneously and synchronously contracting tissue was thus generated and embedded in a microfluidic perfusion bioreactor to create a myocardium-on-a-chip for evaluating cardiotoxicity. The endothelialized microtissue demonstrated dose-dependent reduction in beating rate to the common anticancer drug doxorubicin comparable to prior studies (Zhang et al., 2016). A similar fabrication method and cardiotoxicity test was performed on endothelialized human iPSC-derived microtissue, with results that corresponded well to those observed in the rat-derived microtissue, suggesting translational potential for personalized drug screening (Zhang et al., 2016).

DLP-based 3D printing is a promising approach for the rapid fabrication of cardiac tissues models for the evaluation of potential therapeutics. Ma et al. developed a microscale force gauge array that can be used to align iPSC-CMs and measure the effect of various stimuli including therapeutics, mechanical stretching, electrical stimulation, and calcium on the force output of printed cells (Ma et al., 2019). As force is the key outcome for CM function, monitoring this variable in response to stimuli is a valuable marker of therapeutic efficacy. This 3D model demonstrated improved expression of mature cardiac genes involved in sarcomere force generation, gap junction, and calcium ion channel formation compared to a traditional 2D approach. Liu et al. further developed this model using neonatal mouse ventricular cardiomyocytes (Liu et al., 2020). This study demonstrated that anisotropic 3D patterning of CMs in this microscale force gauge array affects output force generation by 4–10 times when compared to less anisotropically patterned constructs. Importantly, this study demonstrated that CMs directly printed in lines between the pillars of the scaffold had increased function (displacement of the pillars) compared to a slab of cells printed between the pillars. This highlights how DLP-based 3D printing can be used to accurately replicate key features of tissue physiology related to tissue function.

TPP has also been utilized to fabricate filamentous scaffolds of synthetic (Ma et al., 2014) and natural polymers with micron-scale resolution (Gao, Kupfer, et al., 2017). Zhen et al. exposed a photoresist and produced 5 and 10 μm diameter filaments (Figure 2.3C). When iPSC-CMs were seeded onto the scaffold, they aligned along the vertical filaments and their contraction velocity was analyzed. Both healthy and diseased (long-QT syndrome) cardiomyocytes were observed, and their dose responses to various drugs including caffeine, nifedipine (calcium channel blocker), E4031 (potassium channel blocker), and propranolol (beta-blocker) were recorded (Ma et al., 2014). Gao et al. also used TPP to produce 15 μm wide times 100 μm tall lines of GelMA hydrogels. The direct write was repeated producing a layer-by-layer scaffold. iPSC-CMs were seeded with endothelial and smooth muscle cells at a 2:1:1 ratio on fibronectin and collagen supported by the polymerized scaffold and were analyzed for calcium transients and conduction velocity across the entire scaffold. The samples were implanted on a myocardial infarction mouse model, with 11% cell engraftment and significantly improved ejection fraction and fractional shortening at 4

weeks, demonstrating the potential of 3D-printed scaffolds to recover cardiac function (Gao, Kupfer, et al., 2017).

The platforms that have been developed so far typically only involved one cell type. Integrating other cell types and structures remains a challenge but would further improve the physiological relevance of these models. Another challenge that remains is corresponding data generated by a microscale, organ-on-a-chip platform to a human-scale response. As 3D-printing technology advances in terms of resolution, speed, flexibility, and scalability, so does our ability to control tissue architecture and print cardiomyocytes with high viability.

2.3.2.2 Skeletal Muscle Models

Skeletal muscle is one of the most prevalent tissues in the body, accounting for approximately 40% of human body weight. Skeletal muscle has an inherent capacity to regenerate after injury due to the presence of muscle progenitor cells, which are referred to as satellite cells (Katz, 1961; Mauro, 1961). However, this regenerative capacity can be diminished due to multiple factors including injury, aging, and disease.

Skeletal muscle is a highly organized tissue, whose microstructural organization directly informs whole muscle function (Lieber, 2010). Sarcomeres (the basic force generating unit of muscle) form long contractile cells called myofibrils, which are bundled in ECM, to form larger fascicles that are ultimately assembled to form whole muscle. Thus, when fabricating *in vitro* skeletal muscle models, recapitulating this hierarchical organization is key for accurately replicating tissue function. 3D bioprinting of skeletal muscle has an obvious advantage over traditional biofabrication techniques because the orientation and alignment of skeletal muscle cells can easily be controlled. Most 3D bioprinting studies of skeletal muscle employ extrusion 3D bioprinting to form biomimetic muscle structures from cell-laden bioinks. These bioinks typically consist of natural and synthetic biomaterials including PEG, fibrinogen, dECM, GelMA, and PCL to provide structural support during fabrication (Potyondy et al., 2020). While most models use C2C12 mouse muscle progenitor cells, a few studies have also incorporated human muscle progenitor cells (Potyondy et al., 2020).

The majority of 3D-bioprinted skeletal muscle models have been fabricated as potential bioengineered therapies to repair volumetric muscle loss injuries, which can occur due to trauma or surgical ablation (Ahuja et al., 2020; Choi et al., 2019; Gilbert-Honick et al., 2020). These models seek to either stimulate or replace the native regenerative response of skeletal muscle to injury when the injury is too large for it to naturally heal on its own (Gilbert-Honick & Grayson, 2020). However, 3D bioprinting has also been used to study key components of skeletal muscle microstructure and function. For example, Merceron et al. utilized a 3D extrusion bioprinter to replicate a muscle-tendon unit construct using four different components; the muscle side consisted of thermoplastic polyurethane coprinted with C2C12 cells and the tendon side consisted of poly(ϵ -caprolactone) coprinted with 3T3 tenocytes (Merceron et al., 2015). The resulting construct was elastic on the muscle side, stiff on the tendon side, and intermediate at the interface, demonstrating how their 3D-printing technology can be used to fabricate structures with complex, heterogeneous material properties, and cell types. This model of the myotendinous junction can be used to evaluate therapeutics such as platelet rich plasma, where the biological response to treatment is still not well known. Another key component of muscle function stems from the neuromuscular junction, the synapse between a motor neuron and a muscle fiber that allows for the neuron to signal to the muscle fiber

to contract. Using a coaxial 3D bioprinting head, Sanz et al. printed neural and muscle cell cultures which by 14 days in culture exhibited neurite outgrowth and branching, aligned contracting myofibers, and the formation of neuromuscular junctions (Sanz et al., 2020). This model holds promise as few neuromuscular junction systems exist currently for drug evaluation or toxicity testing *in vitro*. Lastly, to accurately replicate the hierarchical structure of skeletal muscle, 3D bioprinting can be used to fabricate scaffolds with physiology informed geometry. Berry et al. used DLP-based 3D printing to fabricate scaffolds with geometry extracted from histology of normal and surgically denervated skeletal muscle (Berry et al., 2017). This technique can be further applied to incorporate physiologically accurate geometry into 3D-bioprinted models of skeletal muscle.

Despite their prevalence, small animal models of skeletal muscle disease and injury do not adequately replicate the same phenotype as found in humans. There is currently a tremendous need for physiologically relevant and biologically accurate *in vitro* models for human myopathies. Advancements in this field can be achieved through the use of patient-derived primary or iPSC cells to provide personalized platforms for drug screening. Key components of skeletal muscle new models of skeletal muscle disease and injury must replicate to be physiologically accurate include muscle fiber alignment, innervation, vascularization, ECM deposition, and the organization of skeletal muscle into its natural, hierarchical structure. Furthermore, similar to the cardiomyocyte studies discussed above, the ability to measure output force in these 3D-bioprinted constructs is a key outcome measure that should be considered during design and testing phases.

2.3.3 CANCER MODELS

Conventional 2D cancer models cultured *in vitro* have provided many essential insights into cancer and led to some key therapeutic successes (McMillin, Negri, & Mitsiades, 2013). However, 2D monolayer models cannot replicate the tumor microenvironment (TME) of native tumors (Leonard & Godin, 2016). TME is highly complex and heterogeneous, and its features including mechanical stimulation, biochemical gradients, and stromal/ECM organization (Albritton & Miller, 2017) affect cancer progression and metastasis through numerous interactions with cancer cells. Tumor–stroma interactions and tumor–ECM interactions are increasingly recognized as factors that influence the treatment responses of tumors to various drugs. These impacts on tumor drug response include ECM or stroma-induced drug resistance and stroma-induced synthetic lethality (McMillin et al., 2010). Drug development for many solid tumors has been stagnant for decades, with over 95% drugs failed during clinical trials (Unger et al., 2014), indicating urgent needs of predictive preclinical models. Metastatic progression, another critical feature of cancer, has been verified to have led to 90% of cancer deaths (Chaffer & Weinberg, 2011), as well as known to be linked with a significant decrease in 5-year survival rates—an important indicator of cancer prognosis and potential treatments. The advancements in 3D bioprinting techniques has given rise to *in vitro* cancer models that better recapitulate TMEs, which subsequently enables better biomimetic investigation of tumor proliferation, invasion, metastasis, response to drugs, as well as tumor–stroma and tumor–ECM interactions. Various cancer models have been fabricated using different bioprinting methods.

Tang and colleagues utilized DLP-based bioprinting to generate patient-derived, multicellular glioblastoma (GBM) models (Tang et al., 2020). GBM is the most common and lethal primary brain cancer that has a dismal 5-year survival rate below 7% in the United States (Ostrom et al., 2019). Difficulty treating GBM is associated with high inter- and inpatient heterogeneity and

immunosuppressive microenvironment. In the bioprinted GBM models, GBM stem cells (GSCs), macrophages, astrocytes, and neural progenitor cells were combined to create a tumor-parenchyma model (Figure 2.4A) (Tang et al., 2020). GSCs in the bioprinted models displayed higher migrative capacity and enhanced resistance to temozolomide and EGFR inhibitors. Monocytes, progenitor cells to macrophages, encapsulated in the bioprinted TME exhibited spontaneous polarization toward the M2 pro-tumor phenotype, indicating TME impacted the stromal cells as well. Incorporation of CRISPR-Cas9 edited GSCs in the bioprinted models demonstrated the potential to use 3D-printed tumor models as novel target discovery platforms. Another bioprinting enabled GBM modeling explored the biophysical impact on tumor progression and drug responses by modulating the mechanical property of matrix materials (Figure 2.4B) (Tang et al., 2021). The GBM model was developed using HA-based bioinks and GSCs. GSCs in the stiffer model which mimicked the pathological stiffness of tumor tissue better recapitulated the transcriptional profile of the mesenchymal subtype of GBM patients. GSCs in the softer model which mimicked the normal brain stiffness resembled the transcriptional profile of the classical or proneural subtypes of GBM patients. GSCs also displayed different migration patterns under the two different stiffness conditions. 3D bioprinting, with its capacity to orthogonally control the biophysical and the biochemical properties of the cell-encapsulating biomaterial matrix, thus has great potential to be used for fabricating models of the highly heterogeneous TMEs.

Extrusion-based bioprinting has also been utilized to investigate the impact of TME on cancer cell proliferation and various tumor characteristics. Zhao et al. reported constructing a $10\text{ mm} \times 10\text{ mm} \times 2\text{ mm}$ grid cervical tumor model with Hela cells in a hydrogel mixture of gelatin, alginate, and fibrinogen (Zhao et al., 2014). The bioprinted cervical tumor model better mimicked the native microenvironment, and the tumor cells exhibited higher proliferation rate and higher simulated tumor characteristics, including matrix metalloproteinase protein expression and chemoresistance against the anticancer treatment paclitaxel, than 2D controls.

To study tumor–stroma interactions, Xu and colleagues used droplet-based printing technique to pattern human ovarian cancer cells and fibroblasts onto a Matrigel substrate (Xu et al., 2011). The printing system consisted of micron-resolution XYZ stages, nanoscale dispensing valves, and $150\text{-}\mu\text{m}$ diameter nozzles. The printed OVCAR-5 with coculture of the fibroblasts proliferated and formed 3D acinar structures that resembled the ovarian cancer micronodules. The results demonstrated that patterning cancer cells with normal stromal cells could enable generation of more physiologically relevant tumor models for better understanding of the cancer mechanisms. 3D-printing technology can also be employed to build special tumor models to evaluate novel therapeutic formulations *in vivo*. For instance, Yang et al. used 3D-printing technology to build a subcutaneous glioblastoma xenograft that mimics the resection tumor cavity (Yang et al., 2017). This tumor model was then used to evaluate the efficiency of a customized drug-releasable implant in preventing glioblastoma recurrence after surgery (Yang et al., 2017).

Incorporation of 3D bioprinting technology with cancer research has demonstrated great potentials in providing reproducible and physiologically relevant tools for mechanistic studies or drug screening. While organoid and patient-derived xenografts have been employed to suggest clinical treatment plans, 3D-printed cancer models with improved reproducibility and highly controllable material and cellular composition can potentially serve as promising *in vitro* drug-sensitivity screening platforms that may benefit cancer patients. More investigation and validation of 3D-printed cancer models including how closely their gene expression, transcriptional profiles, protein

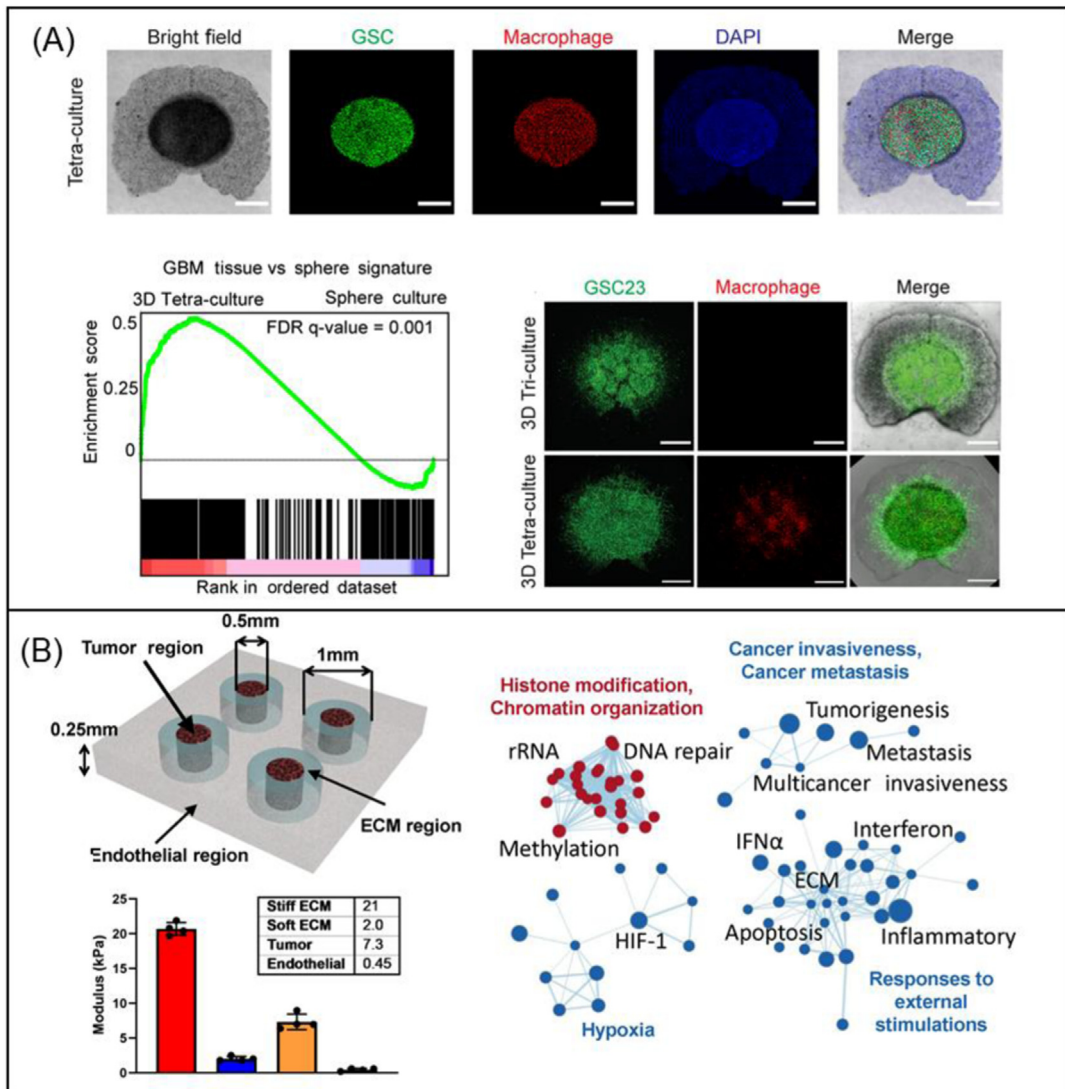


FIGURE 2.4

3D bioprinting of cancer models: (A) schematic diagram showing the design of a multicellular glioblastoma model consisting of tumor cells, macrophages, astrocytes, and neural progenitor cells. Scale bar are 10 μ m. Gene set enrichment analysis demonstrating 3D-printed models recapitulate patient transcriptional profiles better than sphere culture. Fluorescence imaging showing macrophage enhances cancer invasion. (B) Schematic illustration of a glioblastoma-angiogenesis model with regionally varied mechanical properties. Bar plot showing regional stiffnesses of the models. Cytoscape plot from gene set enrichment analysis showing different types of model enrich different pathways.

(A) Reprinted from [Tang et al. \(2020\)](#); (B) Reprinted from [Tang et al. \(2021\)](#).

expression, or functional properties match that of patients will be necessary before their clinical applications can be fully explored.

2.4 CHALLENGES AND FUTURE OUTLOOK

3D bioprinting allows for the precise positioning of biomaterials and living cells to reconstruct complex structures that can be used for disease modeling and drug screening. In each of the fields of liver, heart, muscle, vascular structure, and cancer, researchers have used this technology to build tissue models with organ-specific functions for drug testing applications and for potential tissue implantation. Despite the recent achievements in this field, challenges remain with respect to the printing platforms, cells, and materials used to build tissue models to fully recapitulate the cellular organization and structural complexity of native tissues.

Technological challenges with regard to 3D-printing platforms include the need for increased resolution, printing speed, biocompatibility, and scaling-up. Currently only light-based bioprinters can achieve microscale resolution, which also depends on the type of material used and the cell concentration in the printing mixture. Higher printing resolution is still in great demand to produce complex single cell structures like capillary networks and blastocyst cavities. Higher printing speed also remains an essential challenge for printing organ level structure. The viability of cells in printing solution decreases as printing time increases, particularly for metabolically active cell types like liver and muscle cells. The biocompatibility of 3D-printing platforms has been reported to be satisfactory in the aspects of cell viability, but the impacts on the gene expression and functional aspects are largely understudied. Depending on the type of bioprinter used, there are various mechanical, chemical, and optical disturbances to the printed cells. Further studies on the mechanical, chemical, and optical impacts from the bioprinting process will provide more insight into the biocompatibility of 3D-printing process. Lastly, there are still challenges to the scaling-up of 3D-bioprinted tissue constructs. Current reported applications are largely based on small sample sizes. To consistently generate large numbers of tissue models for clinical and commercial applications, future work is needed to standardize the printers, cells, materials as well as the printing process for high throughput applications (Hwang et al., 2020).

There are also limitations on the types of materials used for 3D bioprinting. Due to the requirements on biomaterials to possess specific biological, chemical, and material properties, there are only a few commonly used materials in 3D bioprinting (Rutz, Hyland, Jakus, Burghardt, & Shah, 2015). Efforts have been made to develop multimaterial bioinks for extrusion-based printing approaches (Rutz et al., 2015). More recently, decellularized ECM has also been studied to create printable biomaterials for extrusion-based and light-based bioprinting platforms (Lee et al., 2017). Unlike highly purified forms of ECM components commonly used such as gelatin and collagen bioinks, dECM bioinks containing the heterogeneous constituents of the native ECM and provides an avenue for researchers to fabricate tissue-specific cell-laden constructs with tissue-matched microenvironments. This approach becomes more important as we strive to develop 3D-printed biomimetic tissues because the native ECM plays a critical role in modulating biological activities including cell proliferation, maturation, migration, and differentiation (Crapo et al., 2011). Together, these efforts will lead to the eventual goal of developing 3D-printable and cell-

compatible materials with tunable mechanical, chemical and biological properties to recapitulate the protein composition and native tissue environment of the specific patient at the targeted health stage.

To apply 3D-printed tissue models to personalized drug screening and disease modeling, patient-specific cell sources, including human iPSC-derived cells and primary diseased cells from patients, will be the main focus. Current studies already demonstrated the use of iPSC-derived cells to build various tissue types (Cha et al., 2014; Faulkner-Jones et al., 2015). However, the maturation of differentiated cells to reach the functional level of adult cells still remains a huge challenge in the field. Primary cells directly harvested from patients are very scarce and not widely applied in current work. Research to advance human iPSC differentiation consistency and maturation protocol is widely demanded. Future applications of using primary diseased cells from patients to build coculture or tri-culture platforms will also provide more insights on the development of personalized disease modeling.

While choosing the appropriate combination of 3D bioprinting technology, materials, and cells is essential in developing complex tissues, establishing a physiologically relevant microenvironment with appropriate physical, chemical, and biological features remains as the ultimate goal (Murphy & Atala, 2014). The review of the four specific application areas outlined the efforts of researchers to create such microenvironment for the corresponding tissue types (Bhise et al., 2016; Ma et al., 2014; Ma et al., 2016; Zhang et al., 2016). In particular, physical features including ECM alignment (Lind et al., 2016; Ma et al., 2014; Zhang et al., 2016), tissue microarchitecture (Huang, Qu, Liu, & Chen, 2014; Ma et al., 2016), and ECM stiffness (Lind et al., 2016; Ma et al., 2016) have been considered and mimicked by various groups working on bioprinted cardiac, liver, and vascularized tissues. Chemical features including complex ECM components (Pati et al., 2014; Zhao et al., 2014) and growth factors (Cui, Breitenkamp, Lotz, & D'Lima, 2012) have also been incorporated across various tissue and cancer systems. Integrating biological features such as cellular composition (Ma et al., 2016; Zhang et al., 2016), cytokine interactions from coculture systems (Xu et al., 2011), and vasculature (Ma et al., 2016; Zhu et al., 2017) are also widely adopted. Despite the current attempts to achieve one or a few features of the tissue type, great challenges remain when simultaneously incorporating all physiologically relevant features to recapitulate a specific microenvironment. Future advancements in 3D-printing technology, material development, and cell sourcing will facilitate this process of establishing physiologically relevant physical, chemical, and biological features for any tissue.

3D bioprinting technology provides the possibility to develop *in vitro* tissue models with physiological relevant cell composition, material properties, complex microstructures, and proper vascularization but this is only the front end of development. Further innovations on postprinting culture platforms such as bioreactors and the incorporation of microfluidic devices will be needed to assist functional maturation and maintenance especially for large, vascularized tissue constructs. Along with these developments, technological advancement in imaging systems and analyzing tools will also be in high demand to analyze large tissue constructs. By applying these dynamic systems, the eventual goal of generating a human-on-a-chip can be realized to create a fully integrated platform studying the interdependent effects of multiple miniaturized organs (Eisenstein, 2015). As such, each organ system can be connected via microfluidics networks and used to revolutionize future drug testing by incorporating biosensors to monitor real-time downstream effects on metabolism, pH, and blood flow on various organs in parallel (Eisenstein, 2015). Overall, advancements in both

research and technology in the fields of medicine, engineering, and biology will be needed to solve these challenges to fully realize the potential of 3D bioprinting in developing sophisticated *in vitro* disease models for precision, patient-specific medicine.

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DECLARATION OF INTERESTS

All authors declare no competing interests.

References

- Ahuja, N., Awad, K., Fiedler, M., Aswath, P., Brotto, M., & Varanasi, V. (2020). Preliminary study of in-situ 3D bioprinted nano-silicate biopolymer scaffolds for muscle repair in VML defects. *The FASEB Journal*, 34. Available from <https://doi.org/10.1096/fasebj.2020.34.s1.03514>, 1–1.
- Akkineni, A. R., Ahlfeld, T., Lode, A., & Gelinsky, M. (2016). A versatile method for combining different biopolymers in a core/shell fashion by 3D plotting to achieve mechanically robust constructs. *Biofabrication*, 8, 45001. Available from <https://doi.org/10.1088/1758-5090/8/4/045001>.
- Albritton, J. L., & Miller, J. S. (2017). 3D bioprinting: Improving *in vitro* models of metastasis with heterogeneous tumor microenvironments. *Disease Models & Mechanisms*, 10, 3–14. Available from <https://doi.org/10.1242/dmm.025049>.
- Almeida, C. R., Serra, T., Oliveira, M. I., Planell, J. A., Barbosa, M. A., & Navarro, M. (2014). Impact of 3-D printed PLA- and chitosan-based scaffolds on human monocyte/macrophage responses: Unraveling the effect of 3-D structures on inflammation. *Acta Biomaterialia*, 10, 613–622. Available from <https://doi.org/10.1016/j.actbio.2013.10.035>.
- Andò, B., & Marletta, V. (2016). An all-inkjet printed bending actuator with embedded sensing feature and an electromagnetic driving mechanism. *Actuators*, 5, 21. Available from <https://doi.org/10.3390/act5030021>.
- AnilKumar, S., Allen, S. C., Tasnim, N., Akter, T., Park, S., Kumar, A., ... Joddar, B. (2018). The applicability of furfuryl-gelatin as a novel bioink for tissue engineering applications. *Journal of Biomedical Materials Research, Part B: Applied Biomaterials*. Available from <https://doi.org/10.1002/jbm.b.34123>.
- Apelgren, P., Amoroso, M., Lindahl, A., Brantsing, C., Rotter, N., Gatenholm, P., & Kölby, L. (2017). Chondrocytes and stem cells in 3D-bioprinted structures create human cartilage *in vivo*. *PLoS One*, 12, e0189428. Available from <https://doi.org/10.1371/journal.pone.0189428>.
- Axpe, E., & Oyen, M. L. (2016). Applications of alginate-based bioinks in 3D bioprinting. *International Journal of Molecular Sciences*, 17, E1976. Available from <https://doi.org/10.3390/ijms17121976>.
- Bataller, R., & Brenner, D. (2005). Liver fibrosis. *The Journal of Clinical Investigation*, 115, 209–218. Available from <https://doi.org/10.1172/JCI200524282>.
- Beachley, V. Z., Wolf, M. T., Sadtler, K., Manda, S. S., Jacobs, H., Blatchley, M. R., ... Elisseeff, J. H. (2015). Tissue matrix arrays for high-throughput screening and systems analysis of cell function. *Nature Methods*, 12, 1197–1204. Available from <https://doi.org/10.1038/nmeth.3619>.

- Bell, A., Kofron, M., & Nistor, V. (2015). Multiphoton crosslinking for biocompatible 3D printing of type I collagen. *Biofabrication*, 7, 35007. Available from <https://doi.org/10.1088/1758-5090/7/3/035007>.
- Benning, L., Gutzweiler, L., Tröndle, K., Riba, J., Zengerle, R., Koltay, P., ... Finkenzeller, G. (2018). Assessment of hydrogels for bioprinting of endothelial cells. *Journal of Biomedical Materials Research Part A*, 106, 935–947. Available from <https://doi.org/10.1002/jbm.a.36291>.
- Berger, D. R., Ware, B. R., Davidson, M. D., Allsup, S. R., & Khetani, S. R. (2014). Enhancing the functional maturity of iPSC-derived human hepatocytes via controlled presentation of cell-cell interactions *in vitro*. *Hepatology (Baltimore, MD)*, 1–44. Available from <https://doi.org/10.1002/hep.27621>.
- Berry, D. B., You, S., Warner, J., Frank, L. R., Chen, S., & Ward, S. R. (2017). A 3D tissue-printing approach for validation of diffusion tensor imaging in skeletal muscle. *Tissue Engineering, Part A*, 23, 980–988. Available from <https://doi.org/10.1089/ten.tea.2016.0438>.
- Bertlein, S., Brown, G., Lim, K. S., Jungst, T., Boeck, T., Blunk, T., ... Groll, J. (2017). Thiol-ene clickable gelatin: A platform bioink for multiple 3D biofabrication technologies. *Advanced Materials*, 29, 1703404. Available from <https://doi.org/10.1002/adma.201703404>.
- Bhatia, S. N., Underhill, G. H., Zaret, K. S., & Fox, I. J. (2014). Cell and tissue engineering for liver disease. *Science Translational Medicine*, 6, 245sr2. Available from <https://doi.org/10.1126/scitranslmed.3005975>.
- Bhise, N. S., Manoharan, V., Massa, S., Tamayol, A., Ghaderi, M., Miscuglio, M., ... Khademhosseini, A. (2016). A liver-on-a-chip platform with bioprinted hepatic spheroids. *Biofabrication*, 8, 014101. Available from <https://doi.org/10.1088/1758-5090/8/1/014101>.
- Bhuthalingam, R., Lim, P. Q., Irvine, S. A., & Venkatraman, S. S. (2016). Automated robotic dispensing technique for surface guidance and bioprinting of cell. *Journal of Visualized Experiments*. Available from <https://doi.org/10.3791/54604>.
- Bourget, J.-M., Kérouédan, O., Medina, M., Rémy, M., Thébaud, N. B., Bareille, R., ... Devillard, R. (2016). Patterning of endothelial cells and mesenchymal stem cells by laser-assisted bioprinting to study cell migration. *Biomed Research International*, 2016, 1–7. Available from <https://doi.org/10.1155/2016/3569843>.
- Burks, H. E., Phamduy, T. B., Azimi, M. S., Saksena, J., Burow, M. E., Collins-Burow, B. M., ... Murfee, W. L. (2016). Laser direct-write onto live tissues: A novel model for studying cancer cell migration. *Journal of Cellular Physiology*, 231, 2333–2338. Available from <https://doi.org/10.1002/jcp.25363>.
- Byambaa, B., Annabi, N., Yue, K., Trujillo-de Santiago, G., Alvarez, M. M., Jia, W., ... Khademhosseini, A. (2017). Bioprinted osteogenic and vasculogenic patterns for engineering 3D bone tissue. *Advanced Healthcare Materials*, 6, 1700015. Available from <https://doi.org/10.1002/adhm.201700015>.
- Cadau, S., Rival, D., Andre-Frei, V., Chavan M, M., Fayol, D., Salducci, M., ... Guillemot, F. (2017). New bioprinted skin, cosmetic *in vitro* model. *Journal of Cosmetic Science*, 68, 85–90.
- Caddeo, S., Boffito, M., & Sartori, S. (2017). Tissue engineering approaches in the design of healthy and pathological *in vitro* tissue models. *Frontiers in Bioengineering and Biotechnology*, 5, 40. Available from <https://doi.org/10.3389/fbioe.2017.00040>.
- Carmen, J., Burger, S. R., McCaman, M., & Rowley, J. A. (2012). Developing assays to address identity, potency, purity and safety: Cell characterization in cell therapy process development. *Regenerative Medicine*, 7, 85–100. Available from <https://doi.org/10.2217/rme.11.105>.
- Cha, C., Soman, P., Zhu, W., Nikkiah, M., Camci-Unal, G., Chen, S., & Khademhosseini, A. (2014). Structural reinforcement of cell-laden hydrogels with microfabricated three dimensional scaffolds. *Biomaterials Science*, 2, 703–709. Available from <https://doi.org/10.1039/C3BM60210A>.
- Chaffer, C. L., & Weinberg, R. A. (2011). A perspective on cancer cell metastasis. *Science (New York, N.Y.)*, 331, 1559–1564. Available from <https://doi.org/10.1126/science.1203543>.
- Chaudhuri, R., Ramachandran, M., Moharil, P., Harumalani, M., & Jaiswal, A. K. (2017). Biomaterials and cells for cardiac tissue engineering: Current choices. *Materials Science and Engineering C*, 79, 950–957. Available from <https://doi.org/10.1016/j.msec.2017.05.121>.

- Chimene, D., Peak, C. W., Gentry, J. L., Carrow, J. K., Cross, L. M., Mondragon, E., ... Gaharwar, A. K. (2018). Nanoengineered ionic-covalent entanglement (NICE) bioinks for 3D bioprinting. *ACS Applied Materials & Interfaces*, 10, 9957–9968. Available from <https://doi.org/10.1021/acsami.7b19808>.
- Choi, Y.-J., Jun, Y.-J., Kim, D. Y., Yi, H.-G., Chae, S.-H., Kang, J., ... Cho, D.-W. (2019). A 3D cell printed muscle construct with tissue-derived bioink for the treatment of volumetric muscle loss. *Biomaterials*, 206, 160–169. Available from <https://doi.org/10.1016/j.biomaterials.2019.03.036>.
- Christensen, K., Xu, C., Chai, W., Zhang, Z., Fu, J., & Huang, Y. (2015). Freeform inkjet printing of cellular structures with bifurcations. *Biotechnology and Bioengineering*, 112, 1047–1055. Available from <https://doi.org/10.1002/bit.25501>.
- Ciociola, A. A., Cohen, L. B., & Kulkarni, P. FDA-Related Matters Committee of the American College of Gastroenterology. (2014). How drugs are developed and approved by the FDA: Current process and future directions. *The American Journal of Gastroenterology*, 109, 620–623.
- Cochis, A., Bonetti, L., Sorrentino, R., Contessi Negrini, N., Grassi, F., Leigheb, M., ... Farè, S. (2018). 3D printing of thermo-responsive methylcellulose hydrogels for cell-sheet engineering. *Materials (Basel)*, 11, 579. Available from <https://doi.org/10.3390/ma11040579>.
- Collins, S. F. (2014). Bioprinting is changing regenerative medicine forever. *Stem Cells and Development*, 23, 79–82. Available from <https://doi.org/10.1089/scd.2014.0322>.
- Colosi, C., Shin, S. R., Manoharan, V., Massa, S., Costantini, M., Barbetta, A., ... Khademhosseini, A. (2016). Microfluidic bioprinting of heterogeneous 3D tissue constructs using low-viscosity bioink. *Advanced Materials*, 28, 677–684. Available from <https://doi.org/10.1002/adma.201503310>.
- Crapo, P. M., Gilbert, T. W., & Badylak, S. F. (2011). An overview of tissue and whole organ decellularization processes. *Biomaterials*, 32, 3233–3243. Available from <https://doi.org/10.1016/j.biomaterials.2011.01.057>.
- Cui, H., Zhu, W., Holmes, B., & Zhang, L. G. (2016). Biologically inspired smart release system based on 3D bioprinted perfused scaffold for vascularized tissue regeneration. *Advanced Science*, 3, 1600058. Available from <https://doi.org/10.1002/adv.201600058>.
- Cui, X., Boland, T., D'Lima, D. D., & Lotz, M. K. (2012). Thermal inkjet printing in tissue engineering and regenerative medicine. *Recent Patents on Drug Delivery & Formulation*, 6, 149–155. Available from <https://doi.org/10.2174/187221112800672949>.
- Cui, X., Breitenkamp, K., Lotz, M., & D'Lima, D. (2012). Synergistic action of fibroblast growth factor-2 and transforming growth factor-beta1 enhances bioprinted human neocartilage formation. *Biotechnology and Bioengineering*, 109, 2357–2368. Available from <https://doi.org/10.1002/bit.24488>.
- Cumpston, B. H., Ananthavel, S. P., Barlow, S., Dyer, D. L., Ehrlich, J. E., Erskine, L. L., ... Perry, J. W. (1999). Two-photon polymerization initiators for three-dimensional optical data storage and microfabrication. *Nature*, 398, 51–54. Available from <https://doi.org/10.1038/17989>.
- Dai, X., Liu, L., Ouyang, J., Li, X., Zhang, X., Lan, Q., & Xu, T. (2017). Coaxial 3D bioprinting of self-assembled multicellular heterogeneous tumor fibers. *Scientific Reports*, 7, 1457. Available from <https://doi.org/10.1038/s41598-017-01581-y>.
- Di Bella, C., Duchi, S., O'Connell, C. D., Blanchard, R., Augustine, C., Yue, Z., ... Choong, P. F. (2018). *In situ* handheld three-dimensional bioprinting for cartilage regeneration. *Journal of Tissue Engineering and Regenerative Medicine*, 12, 611–621. Available from <https://doi.org/10.1002/term.2476>.
- Di Giuseppe, M., Law, N., Webb, B., Macrae, R. A., Liew, L. J., Sercombe, T. B., ... Doyle, B. J. (2018). Mechanical behaviour of alginate-gelatin hydrogels for 3D bioprinting. *Journal of the Mechanical Behavior of Biomedical Materials*, 79, 150–157. Available from <https://doi.org/10.1016/j.jmbbm.2017.12.018>.
- Diamantides, N., Wang, L., Pruiksma, T., Siemiatkoski, J., Dugopolski, C., Shortkroff, S., ... Bonassar, L. J. (2017). Correlating rheological properties and printability of collagen bioinks: The effects of riboflavin

- photocrosslinking and pH. *Biofabrication*, 9, 34102. Available from <https://doi.org/10.1088/1758-5090/aa780f>.
- Dinh, N.-D., Luo, R., Tankeh, M., Christine, A., Lin, W. N., Shih, W.-C., . . . Hong Goh, C.-H. (2017). Chen, Effective light directed assembly of building blocks with microscale control. *Small (Weinheim an der Bergstrasse, Germany)*, 13. Available from <https://doi.org/10.1002/smll.201700684>.
- Domansky, K., Inman, W., Serdy, J., Dash, A., Lim, M. H. M., & Griffith, L. G. (2010). Perfused multiwell plate for 3D liver tissue engineering. *Lab on a Chip*, 10, 51–58. Available from <https://doi.org/10.1039/b913221j>.
- Du, Y., Han, R., Wen, F., Ng San San, S., Xia, L., Wohland, T., . . . Yu, H. (2008). Synthetic sandwich culture of 3D hepatocyte monolayer. *Biomaterials*, 29, 290–301. Available from <https://doi.org/10.1016/j.biomaterials.2007.09.016>.
- Dubbin, K., Tabet, A., & Heilshorn, S. C. (2017). Quantitative criteria to benchmark new and existing bio-inks for cell compatibility. *Biofabrication*, 9, 44102. Available from <https://doi.org/10.1088/1758-5090/aa869f>.
- Duchi, S., Onofrillo, C., O'Connell, C. D., Blanchard, R., Augustine, C., Quigley, A. F., . . . Choong, P. F. M. (2017). Handheld co-axial bioprinting: Application to *in situ* surgical cartilage repair. *Scientific Reports*, 7, 5837. Available from <https://doi.org/10.1038/s41598-017-05699-x>.
- Eisenstein, M. (2015). Artificial organs: Honey, I shrunk the lungs. *Nature*, 519, S16–S18. Available from <https://doi.org/10.1038/519S16a>.
- Engelhardt, S., Hoch, E., Borchers, K., Meyer, W., Krüger, H., Tovar, G. E. M., & Gillner, A. (2011). Fabrication of 2D protein microstructures and 3D polymer–protein hybrid microstructures by two-photon polymerization. *Biofabrication*, 3, 25003. Available from <https://doi.org/10.1088/1758-5082/3/2/025003>.
- Engler, A. J., Carag-Krieger, C., Johnson, C. P., Raab, M., Tang, H.-Y., Speicher, D. W., . . . Discher, D. E. (2008). Embryonic cardiomyocytes beat best on a matrix with heart-like elasticity: Scar-like rigidity inhibits beating. *Journal of Cell Science*, 121, 3794–3802. Available from <https://doi.org/10.1242/jcs.029678>.
- Ersumo, N., Witherel, C. E., & Spiller, K. L. (2016). Differences in time-dependent mechanical properties between extruded and molded hydrogels. *Biofabrication*, 8, 35012. Available from <https://doi.org/10.1088/1758-5090/8/3/035012>.
- Fang, Y., & Eglén, R. M. (2017). Three-dimensional cell cultures in drug discovery and development. *SLAS Discovery: Advancing Life Sciences R & D*, 22, 456–472. Available from <https://doi.org/10.1177/1087057117696795>.
- Farsari, M., & Chichkov, B. N. (2009). Materials processing: Two-photon fabrication. *Nature Photonics*, 3, 450–452. Available from <https://doi.org/10.1038/nphoton.2009.131>.
- Faulkner-Jones, A., Fyfe, C., Cornelissen, D.-J., Gardner, J., King, J., Courtney, A., & Shu, W. (2015). Bioprinting of human pluripotent stem cells and their directed differentiation into hepatocyte-like cells for the generation of mini-livers in 3D. *Biofabrication*, 7, 044102. Available from <https://doi.org/10.1088/1758-5090/7/4/044102>.
- Gao, G., Schilling, A. F., Hubbell, K., Yonezawa, T., Truong, D., Hong, Y., . . . Cui, X. (2015). Improved properties of bone and cartilage tissue from 3D inkjet-bioprinted human mesenchymal stem cells by simultaneous deposition and photocrosslinking in PEG-GelMA. *Biotechnology Letters*, 37, 2349–2355. Available from <https://doi.org/10.1007/s10529-015-1921-2>.
- Gao, G., Yonezawa, T., Hubbell, K., Dai, G., & Cui, X. (2015). Inkjet-bioprinted acrylated peptides and PEG hydrogel with human mesenchymal stem cells promote robust bone and cartilage formation with minimal printhead clogging. *Biotechnology Journal*, 10, 1568–1577. Available from <https://doi.org/10.1002/biot.201400635>.
- Gao, G., Hubbell, K., Schilling, A. F., Dai, G., & Cui, X. (2017). Bioprinting cartilage tissue from mesenchymal stem cells and PEG hydrogel. *Methods in Molecular Biology*, 391–398. Available from https://doi.org/10.1007/978-1-4939-7021-6_28.

- Gao, L., Kupfer, M. E., Jung, J. P., Yang, L., Zhang, P., Da Sie, Y., ... Zhang, J. (2017). Myocardial tissue engineering with cells derived from human-induced pluripotent stem cells and a native-like, high-resolution, 3-dimensionally printed scaffold. *Circulation Research*, 120, 1318–1325. Available from <https://doi.org/10.1161/CIRCRESAHA.116.310277>.
- Gao, Q., He, Y., Fu, J., Liu, A., & Ma, L. (2015). Coaxial nozzle-assisted 3D bioprinting with built-in micro-channels for nutrients delivery. *Biomaterials*, 61, 203–215. Available from <https://doi.org/10.1016/j.biomaterials.2015.05.031>.
- Gaskell, H., Sharma, P., Colley, H. E., Murdoch, C., Williams, D. P., & Webb, S. D. (2016). Characterization of a functional C3A liver spheroid model. *Toxicology Research*, 5, 1053–1065. Available from <https://doi.org/10.1039/c6tx00101g>.
- Gettler, B. C., Zakhari, J. S., Gandhi, P. S., & Williams, S. K. (2017). Formation of adipose stromal vascular fraction cell-laden spheroids using a three-dimensional bioprinter and superhydrophobic surfaces. *Tissue Engineering: Part C, Methods*, 23, 516–524. Available from <https://doi.org/10.1089/ten.TEC.2017.0056>.
- Gi, H. J., Han, D., & Park, J.-K. (2017). Optoelectrofluidic printing system for fabricating hydrogel sheets with on-demand patterned cells and microparticles. *Biofabrication*, 9, 15011. Available from <https://doi.org/10.1088/1758-5090/aa564c>.
- Giannopoulos, A. A., Mitsouras, D., Yoo, S.-J., Liu, P. P., Chatzizisis, Y. S., & Rybicki, F. J. (2016). Applications of 3D printing in cardiovascular diseases. *Nature Reviews Cardiology*, 13, 701–718. Available from <https://doi.org/10.1038/nrcardio.2016.170>.
- Gilbert-Honick, J., & Grayson, W. (2020). Vascularized and innervated skeletal muscle tissue engineering. *Advanced Healthcare Materials*, 9, e1900626. Available from <https://doi.org/10.1002/adhm.201900626>.
- Gilbert-Honick, J., Iyer, S. R., Somers, S. M., Takasuka, H., Lovering, R. M., Wagner, K. R., ... Grayson, W. L. (2020). Engineering 3D skeletal muscle primed for neuromuscular regeneration following volumetric muscle loss. *Biomaterials*, 255, 120154. Available from <https://doi.org/10.1016/j.biomaterials.2020.120154>.
- Gomes, S., Leonor, I. B., Mano, J. F., Reis, R. L., & Kaplan, D. L. (2012). Natural and genetically engineered proteins for tissue engineering. *Progress in Polymer Science*, 37, 1–17. Available from <https://doi.org/10.1016/j.progpolymsci.2011.07.003>.
- Gou, M., Qu, X., Zhu, W., Xiang, M., Yang, J., Zhang, K., ... Chen, S. (2014). Bio-inspired detoxification using 3D-printed hydrogel nanocomposites. *Nature Communications*, 5, 3774. Available from <https://doi.org/10.1038/ncomms4774>.
- Graham, A. D., Olof, S. N., Burke, M. J., Armstrong, J. P. K., Mikhailova, E. A., Nicholson, J. G., ... Bayley, H. (2017). High-resolution patterned cellular constructs by droplet-based 3D printing. *Scientific Reports*, 7, 7004. Available from <https://doi.org/10.1038/s41598-017-06358-x>.
- Gribova, V., Crouzier, T., & Picart, C. (2011). A material's point of view on recent developments of polymeric biomaterials: Control of mechanical and biochemical properties. *Journal of Materials Chemistry*, 21, 14354–14366. Available from <https://doi.org/10.1039/C1JM11372K>.
- Grogan, S. P., Chung, P. H., Soman, P., Chen, P., Lotz, M. K., Chen, S., & D'Lima, D. D. (2013). Digital micromirror device projection printing system for meniscus tissue engineering. *Acta Biomaterialia*, 9, 7218–7226. Available from <https://doi.org/10.1016/j.actbio.2013.03.020>.
- Gu, Q., Tomaskovic-Crook, E., Wallace, G. G., & Crook, J. M. (2017). 3D bioprinting human induced pluripotent stem cell constructs for *in situ* cell proliferation and successive multilineage differentiation. *Advanced Healthcare Materials*, 6, 1700175. Available from <https://doi.org/10.1002/adhm.201700175>.
- Guguen-Guillouzo, C., Corlu, A., & Guillouzo, A. (2010). Stem cell-derived hepatocytes and their use in toxicology. *Toxicology*, 270, 3–9. Available from <https://doi.org/10.1016/j.tox.2009.09.019>.
- Hart, L. R., Li, S., Sturgess, C., Wildman, R., Jones, J. R., & Hayes, W. (2016). 3D printing of biocompatible supramolecular polymers and their composites. *ACS Applied Materials & Interfaces*, 8, 3115–3122. Available from <https://doi.org/10.1021/acsami.5b10471>.

- Hastings, C. L., Roche, E. T., Ruiz-Hernandez, E., Schenke-Layland, K., Walsh, C. J., & Duffy, G. P. (2015). Drug and cell delivery for cardiac regeneration. *Advanced Drug Delivery Reviews*, 84, 85–106. Available from <https://doi.org/10.1016/j.addr.2014.08.006>.
- Hendriks, J., Willem Visser, C., Henke, S., Leijten, J., Saris, D. B. F., Sun, C., ... Karperien, M. (2015). Optimizing cell viability in droplet-based cell deposition. *Scientific Reports*, 5, 11304. Available from <https://doi.org/10.1038/srep11304>.
- Hewitt, N. J., Gómez Lechón, M. J., Houston, J. B., Hallifax, D., Brown, H. S., Maurel, P., ... Hengstler, J. G. (2007). Primary hepatocytes: Current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metabolism Reviews*, 39, 159–234. Available from <https://doi.org/10.1080/03602530601093489>.
- Highley, C. B., Rodell, C. B., & Burdick, J. A. (2015). Direct 3D printing of shear-thinning hydrogels into self-healing hydrogels. *Advanced Materials*, 27, 5075–5079. Available from <https://doi.org/10.1002/adma.201501234>.
- Hirt, M. N., Hansen, A., & Eschenhagen, T. (2014). Cardiac tissue engineering: State of the art. *Circulation Research*, 114, 354–367. Available from <https://doi.org/10.1161/CIRCRESAHA.114.300522>.
- Ho, C.-T., Lin, R.-Z., Chen, R.-J., Chin, C.-K., Gong, S.-E., Chang, H.-Y., ... Liu, C.-H. (2013). Liver-cell patterning lab on a chip: Mimicking the morphology of liver lobule tissue. *Lab on a Chip*, 13, 3578–3587. Available from <https://doi.org/10.1039/C3LC50402F>.
- Ho, L., & Hsu, S. (2018). Cell reprogramming by 3D bioprinting of human fibroblasts in polyurethane hydrogel for fabrication of neural-like constructs. *Acta Biomaterialia*, 70, 57–70. Available from <https://doi.org/10.1016/j.actbio.2018.01.044>.
- Hribar, K. C., Soman, P., Warner, J., Chung, P., & Chen, S. (2014). Light-assisted direct-write of 3D functional biomaterials. *Lab on a Chip*, 14, 268–275. Available from <https://doi.org/10.1039/C3LC50634G>.
- Hribar, K. C., Finlay, D., Ma, X., Qu, X., Ondeck, M. G., Chung, P. H., ... Chen, S. (2015). Nonlinear 3D projection printing of concave hydrogel microstructures for long-term multicellular spheroid and embryoid body culture. *Lab on a Chip*, 15, 2412–2418. Available from <https://doi.org/10.1039/C5LC00159E>.
- Hu, Y., Wu, Y., Gou, Z., Tao, J., Zhang, J., Liu, Q., ... Gou, M. (2016). 3D-engineering of cellularized conduits for peripheral nerve regeneration. *Scientific Reports*, 6, 32184. Available from <https://doi.org/10.1038/srep32184>.
- Huang, J., Fu, H., Li, C., Dai, J., & Zhang, Z. (2017). Recent advances in cell-laden 3D bioprinting: Materials, technologies and applications. *Journal of 3D Printing in Medicine*, 1, 245–268. Available from <https://doi.org/10.2217/3dp-2017-0010>.
- Huang, T. Q., Qu, X., Liu, J., & Chen, S. (2014). 3D printing of biomimetic microstructures for cancer cell migration. *Biomedical Microdevices*, 16, 127–132. Available from <https://doi.org/10.1007/s10544-013-9812-6>.
- Hung, B. P., Naved, B. A., Nyberg, E. L., Dias, M., Holmes, C. A., Elisseeff, J. H., ... Grayson, W. L. (2016). Three-dimensional printing of bone extracellular matrix for craniofacial regeneration. *ACS Biomaterials Science & Engineering*, 2, 1806–1816. Available from <https://doi.org/10.1021/acsbiomaterials.6b00101>.
- Hutson, C. B., Nichol, J. W., Aubin, H., Bae, H., Yamanlar, S., Al-Haque, S., ... Khademhosseini, A. (2011). Synthesis and characterization of tunable poly(ethylene glycol): Gelatin methacrylate composite hydrogels. *Tissue Engineering*, 17, 1713–1723.
- Hwang, H. H., You, S., Ma, X., Kwe, L., Victorine, G., Lawrence, N., ... Chen, S. (2020). High throughput direct 3D bioprinting in multiwell plates. *Biofabrication*. Available from <https://doi.org/10.1088/1758-5090/ab89ca>.
- Hölzl, K., Lin, S., Tytgat, L., Van Vlierberghe, S., Gu, L., & Ovsianikov, A. (2016). Bioink properties before, during and after 3D bioprinting. *Biofabrication*, 8, 032002. Available from <https://doi.org/10.1088/1758-5090/8/3/032002>.

- Inzana, J. A., Olvera, D., Fuller, S. M., Kelly, J. P., Graeve, O. A., Schwarz, E. M., ... Awad, H. A. (2014). 3D printing of composite calcium phosphate and collagen scaffolds for bone regeneration. *Biomaterials*, 35, 4026–4034. Available from <https://doi.org/10.1016/j.biomaterials.2014.01.064>.
- Irvine, S., & Venkatraman, S. (2016). Bioprinting and differentiation of stem cells. *Molecules (Basel, Switzerland)*, 21, 1188. Available from <https://doi.org/10.3390/molecules21091188>.
- Jammalamadaka, U., & Tappa, K. (2018). Recent advances in biomaterials for 3D printing and tissue engineering. *Journal of Functional Biomaterials*, 9, 22. Available from <https://doi.org/10.3390/jfb9010022>.
- Jang, J., Kim, T. G., Kim, B. S., Kim, S.-W., Kwon, S.-M., & Cho, D.-W. (2016). Tailoring mechanical properties of decellularized extracellular matrix bioink by vitamin B2-induced photo-crosslinking. *Acta Biomaterialia*, 33, 88–95. Available from <https://doi.org/10.1016/j.actbio.2016.01.013>.
- Ji, S., & Guvendiren, M. (2017). Recent advances in bioink design for 3D bioprinting of tissues and organs. *Frontiers in Bioengineering and Biotechnology*, 5, 23. Available from <https://doi.org/10.3389/fbioe.2017.00023>.
- Jia, W., Gungor-Ozkerim, P. S., Zhang, Y. S., Yue, K., Zhu, K., Liu, W., ... Khademhosseini, A. (2016). Direct 3D bioprinting of perfusable vascular constructs using a blend bioink. *Biomaterials*, 106, 58–68. Available from <https://doi.org/10.1016/j.biomaterials.2016.07.038>.
- Jin, Y., Compaan, A., Bhattacharjee, T., & Huang, Y. (2016). Granular gel support-enabled extrusion of three-dimensional alginate and cellular structures. *Biofabrication*, 8, 25016. Available from <https://doi.org/10.1088/1758-5090/8/2/025016>.
- Jin, Y., Liu, C., Chai, W., Compaan, A., & Huang, Y. (2017). Self-supporting nanoclay as internal scaffold material for direct printing of soft hydrogel composite structures in air. *ACS Applied Materials & Interfaces*, 9, 17456–17465. Available from <https://doi.org/10.1021/acsami.7b03613>.
- Kang, H.-W., Lee, S. J., Ko, I. K., Kengla, C., Yoo, J. J., & Atala, A. (2016). A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. *Nature Biotechnology*, 34, 312–319. Available from <https://doi.org/10.1038/nbt.3413>.
- Kang, K., Kim, Y., Lee, S. B., Kim, J. S., Park, S., Kim, W., ... Choi, D. (2018). Three-dimensional bio-printing of hepatic structures with direct-converted hepatocyte-like cells. *Tissue Engineering, Part A*, 24, 576–583. Available from <https://doi.org/10.1089/ten.TEA.2017.0161>.
- Kang, L. H., Armstrong, P. A., Lee, L. J., Duan, B., Kang, K. H., & Butcher, J. T. (2017). Optimizing photo-encapsulation viability of heart valve cell types in 3D printable composite hydrogels. *Annals of Biomedical Engineering*, 45, 360–377. Available from <https://doi.org/10.1007/s10439-016-1619-1>.
- Kaplowitz, N. (2005). Idiosyncratic drug hepatotoxicity. *Nature Reviews: Drug Discovery*, 4, 489–499. Available from <https://doi.org/10.1038/nrd1750>.
- Katz, B. (1961). The termination of the afferent nerve fibre in the muscle spindle of the frog. *Philosophical Transactions of the Royal Society of London: Series B, Biological Sciences*, 243, 221–240. Available from <https://doi.org/10.1098/rstb.1961.0001>.
- Kawecki, F., Clafshenkel, W. P., Auger, F. A., Bourget, J.-M., Fradette, J., & Devillard, R. (2018). Self-assembled human osseous cell sheets as living biopapers for the laser-assisted bioprinting of human endothelial cells. *Biofabrication*. Available from <https://doi.org/10.1088/1758-5090/aabd5b>.
- Keane, T. J., & Badylak, S. F. (2014). Biomaterials for tissue engineering applications. *Seminars in Pediatric Surgery*, 23, 112–118. Available from <https://doi.org/10.1053/j.sempedsurg.2014.06.010>.
- Kelly, B. E., Bhattacharya, I., Heidari, H., Shusteff, M., Spadaccini, C. M., & Taylor, H. K. (2019). Volumetric additive manufacturing via tomographic reconstruction. *Science (New York, N.Y.)*, 363, 1075–1079. Available from <https://doi.org/10.1126/science.aau7114>.
- Keriquel, V., Oliveira, H., Rémy, M., Ziane, S., Delmond, S., Rousseau, B., ... Fricain, J.-C. (2017). *In situ* printing of mesenchymal stromal cells, by laser-assisted bioprinting, for *in vivo* bone regeneration applications. *Scientific Reports*, 7, 1778. Available from <https://doi.org/10.1038/s41598-017-01914-x>.

- Kesti, M., Müller, M., Becher, J., Schnabelrauch, M., D'Este, M., Eglin, D., & Zenobi-Wong, M. (2015). A versatile bioink for three-dimensional printing of cellular scaffolds based on thermally and photo-triggered tandem gelation. *Acta Biomaterialia*, 11, 162–172. Available from <https://doi.org/10.1016/j.actbio.2014.09.033>.
- Khetani, S. R., & Bhatia, S. N. (2008). Microscale culture of human liver cells for drug development. *Nature Biotechnology*, 26, 120–126. Available from <https://doi.org/10.1038/nbt1361>.
- Kim, B. S., Jang, J., Chae, S., Gao, G., Kong, J.-S., Ahn, M., & Cho, D.-W. (2016). Three-dimensional bioprinting of cell-laden constructs with polycaprolactone protective layers for using various thermoplastic polymers. *Biofabrication*, 8, 35013. Available from <https://doi.org/10.1088/1758-5090/8/3/035013>.
- Kim, B. S., Lee, J.-S., Gao, G., & Cho, D.-W. (2017). Direct 3D cell-printing of human skin with functional transwell system. *Biofabrication*, 9, 25034. Available from <https://doi.org/10.1088/1758-5090/aa71c8>.
- Kim, J. D., Choi, J. S., Kim, B. S., Chan Choi, Y., & Cho, Y. W. (2010). Piezoelectric inkjet printing of polymers: Stem cell patterning on polymer substrates. *Polymer*, 51, 2147–2154. Available from <https://doi.org/10.1016/j.polymer.2010.03.038>.
- Kim, K., Ohashi, K., Utoh, R., Kano, K., & Okano, T. (2012). Preserved liver-specific functions of hepatocytes in 3D co-culture with endothelial cell sheets. *Biomaterials*, 33, 1406–1413. Available from <https://doi.org/10.1016/j.biomaterials.2011.10.084>.
- Kinarivala, N., Shah, K., Abbruscato, T. J., & Trippier, P. C. (2017). Passage variation of PC12 cells results in inconsistent susceptibility to externally induced apoptosis. *ACS Chemical Neuroscience*, 8, 82–88. Available from <https://doi.org/10.1021/acschemneuro.6b00208>.
- Kizawa, H., Nagao, E., Shimamura, M., Zhang, G., & Torii, H. (2017). Scaffold-free 3D bio-printed human liver tissue stably maintains metabolic functions useful for drug discovery. *Biochemistry and Biophysics Reports*, 10, 186–191. Available from <https://doi.org/10.1016/j.bbrep.2017.04.004>.
- Koch, L., Deiwick, A., Franke, A., Schwanke, K., Haverich, A., Zweigerdt, R., & Chichkov, B. N. (2018). Laser bioprinting of human induced pluripotent stem cells – The effect of printing and biomaterials on cell survival, pluripotency, and differentiation. *Biofabrication*. Available from <https://doi.org/10.1088/1758-5090/aab981>.
- Kolesky, D. B., Truby, R. L., Gladman, A. S., Busbee, T. A., Homan, K. A., & Lewis, J. A. (2014). 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. *Advanced Materials*, 26, 3124–3130. Available from <https://doi.org/10.1002/adma.201305506>.
- Kolesky, D. B., Homan, K. A., Skylar-Scott, M. A., & Lewis, J. A. (2016). Three-dimensional bioprinting of thick vascularized tissues. *Proceedings of the National Academy of Sciences of the United States of America*, 113, 3179–3184. Available from <https://doi.org/10.1073/pnas.1521342113>.
- Kollamaram, G., Hopkins, S. C., Glowacki, B. A., Croker, D. M., & Walker, G. M. (2018). Inkjet printing of paracetamol and indomethacin using electromagnetic technology: Rheological compatibility and polymorphic selectivity. *European Journal of Pharmaceutical Sciences*, 115, 248–257. Available from <https://doi.org/10.1016/j.ejps.2018.01.036>.
- Koo, Y., & Kim, G. (2016). New strategy for enhancing *in situ* cell viability of cell-printing process via piezoelectric transducer-assisted three-dimensional printing. *Biofabrication*, 8, 25010. Available from <https://doi.org/10.1088/1758-5090/8/2/025010>.
- Laronda, M. M., Rutz, A. L., Xiao, S., Whelan, K. A., Duncan, F. E., Roth, E. W., ... Shah, R. N. (2017). A bioprosthetic ovary created using 3D printed microporous scaffolds restores ovarian function in sterilized mice. *Nature Communications*, 8, 15261. Available from <https://doi.org/10.1038/ncomms15261>.
- Leberfinger, A. N., Ravnic, D. J., Dhawan, A., & Ozbolat, I. T. (2017). Concise review: Bioprinting of stem cells for transplantable tissue fabrication. *Stem Cells Translational Medicine*, 6, 1940–1948. Available from <https://doi.org/10.1002/sctm.17-0148>.

- Lee, H., Yoo, J. J., Kang, H.-W., & Cho, D.-W. (2016). Investigation of thermal degradation with extrusion-based dispensing modules for 3D bioprinting technology. *Biofabrication*, 8, 15011. Available from <https://doi.org/10.1088/1758-5090/8/1/015011>.
- Lee, H., Han, W., Kim, H., Ha, D.-H., Jang, J., Kim, B. S., & Cho, D.-W. (2017). Development of liver decellularized extracellular matrix bioink for three-dimensional cell printing-based liver tissue engineering. *Biomacromolecules*, 18, 1229–1237. Available from <https://doi.org/10.1021/acs.biomac.6b01908>.
- Lee, J. M., & Yeong, W. Y. (2016). Design and printing strategies in 3D bioprinting of cell-hydrogels: A review. *Advanced Healthcare Materials*, 5, 2856–2865. Available from <https://doi.org/10.1002/adhm.201600435>.
- Lee, J. W., Soman, P., Park, J. H., Chen, S., & Cho, D. W. (2016). A tubular biomaterial construct exhibiting a negative Poisson's ratio. *PLoS One*, 11, 1–14. Available from <https://doi.org/10.1371/journal.pone.0155681>.
- Legant, W. R., Pathak, A., Yang, M. T., Deshpande, V. S., McMeeking, R. M., & Chen, C. S. (2009). Microfabricated tissue gauges to measure and manipulate forces from 3D microtissues. *Proceedings of the National Academy of Sciences*, 106, 10097–10102. Available from <https://doi.org/10.1073/pnas.0900174106>.
- Leonard, F., & Godin, B. (2016). 3D *in vitro* model for breast cancer research using magnetic levitation and bioprinting method. *Methods in Molecular Biology*, 1406, 239–251. Available from https://doi.org/10.1007/978-1-4939-3444-7_21.
- Li, J., Chen, M., Fan, X., & Zhou, H. (2016). Recent advances in bioprinting techniques: Approaches, applications and future prospects. *Journal of Translational Medicine*, 14, 271. Available from <https://doi.org/10.1186/s12967-016-1028-0>.
- Li, Y.-C., Zhu, K., & Young, T.-H. (2017). Induced pluripotent stem cells, form *in vitro* tissue engineering to *in vivo* allogeneic transplantation. *Journal of Thoracic Disease*, 9, 455–459. Available from <https://doi.org/10.21037/jtd.2017.02.77>.
- Lieber, R. L. (2010). *Skeletal muscle structure, function, and plasticity: The physiological basis of rehabilitation* (3rd ed.). Baltimore, MD: Lippincott Williams & Wilkins.
- Lind, J. U., Busbee, T. A., Valentine, A. D., Pasqualini, F. S., Yuan, H., Yadid, M., . . . Parker, K. K. (2016). Instrumented cardiac microphysiological devices via multimaterial three-dimensional printing. *Nature Materials*, 16, 303–308. Available from <https://doi.org/10.1038/nmat4782>.
- Liu, J., Hwang, H. H., Wang, P., Whang, G., & Chen, S. (2016). Direct 3D-printing of cell-laden constructs in microfluidic architectures. *Lab on a Chip*, 16, 1430–1438. Available from <https://doi.org/10.1039/c6lc00144k>.
- Liu, J., Miller, K., Ma, X., Dewan, S., Lawrence, N., Whang, G., . . . Chen, S. (2020). Direct 3D bioprinting of cardiac micro-tissues mimicking native myocardium. *Biomaterials*, 256, 120204. Available from <https://doi.org/10.1016/j.biomaterials.2020.120204>.
- Liu, P., Kaplan, A., Yuan, B., Hanna, J. H., Lupski, J. R., & Reiner, O. (2014). Passage number is a major contributor to genomic structural variations in mouse iPSCs. *Stem Cells*, 32, 2657–2667. Available from <https://doi.org/10.1002/stem.1779>.
- Liu, W., Heinrich, M. A., Zhou, Y., Akpek, A., Hu, N., Liu, X., . . . Zhang, Y. S. (2017). Extrusion bioprinting of shear-thinning gelatin methacryloyl bioinks. *Advanced Healthcare Materials*, 6, 1601451. Available from <https://doi.org/10.1002/adhm.201601451>.
- Liu, W., Zhang, Y. S., Heinrich, M. A., De Ferrari, F., Jang, H. L., Bakht, S. M., . . . Khademhosseini, A. (2017). Rapid continuous multimaterial extrusion bioprinting. *Advanced Materials*, 29. Available from <https://doi.org/10.1002/adma.201604630>.
- Liu, W., Zhong, Z., Hu, N., Zhou, Y., Maggio, L., Miri, A. K., . . . Zhang, Y. S. (2018). Coaxial extrusion bioprinting of 3D microfibrous constructs with cell-favorable gelatin methacryloyl microenvironments. *Biofabrication*, 10, 24102. Available from <https://doi.org/10.1088/1758-5090/aa9d44>.

- Lu, L., Peter, S. J., Lyman, M. D., Lai, H. L., Leite, S. M., Tamada, J. A., . . . Mikos, A. G. (2000). *In vitro* and *in vivo* degradation of porous poly(DL-lactic-co-glycolic acid) foams. *Biomaterials*, 21, 1837–1845.
- Lundy, S. D., Gantz, J. A., Pagan, C. M., Filice, D., & Laflamme, M. A. (2014). Pluripotent stem cell derived cardiomyocytes for cardiac repair. *Current Treatment Options in Cardiovascular Medicine*, 16, 319. Available from <https://doi.org/10.1007/s11936-014-0319-0>.
- Ma, X., Qu, X., Zhu, W., Li, Y.-S., Yuan, S., Zhang, H., . . . Chen, S. (2016). Deterministically patterned bio-mimetic human iPSC-derived hepatic model via rapid 3D bioprinting. *Proceedings of the National Academy of Sciences*, 113, 2206–2211. Available from <https://doi.org/10.1073/pnas.1524510113>.
- Ma, X., Yu, C., Wang, P., Xu, W., Wan, X., Lai, C. S. E., . . . Chen, S. (2018). Rapid 3D bioprinting of decellularized extracellular matrix with regionally varied mechanical properties and biomimetic microarchitecture. *Biomaterials*, 185, 310–321. Available from <https://doi.org/10.1016/j.biomaterials.2018.09.026>.
- Ma, X., Dewan, S., Liu, J., Tang, M., Miller, K. L., Yu, C., . . . Chen, S. (2019). 3D printed micro-scale force gauge arrays to improve human cardiac tissue maturation and enable high throughput drug testing. *Acta Biomaterialia*, 95, 319–327. Available from <https://doi.org/10.1016/j.actbio.2018.12.026>.
- Ma, Z., Koo, S., Finnegan, M. A., Loskill, P., Huebsch, N., Marks, N. C., . . . Healy, K. E. (2014). Three-dimensional filamentous human diseased cardiac tissue model. *Biomaterials*, 35, 1367–1377. Available from <https://doi.org/10.1016/j.biomaterials.2013.10.052>.
- Maiullari, F., Costantini, M., Milan, M., Pace, V., Chirivì, M., Maiullari, S., . . . Rizzi, R. (2018). A multi-cellular 3D bioprinting approach for vascularized heart tissue engineering based on HUVECs and iPSC-derived cardiomyocytes. *Scientific Reports*, 8, 13532. Available from <https://doi.org/10.1038/s41598-018-31848-x>.
- Markstedt, K., Mantas, A., Tournier, I., Martínez Ávila, H., Hägg, D., & Gatenholm, P. (2015). 3D bioprinting human chondrocytes with nanocellulose–alginate bioink for cartilage tissue engineering applications. *Biomacromolecules*, 16, 1489–1496. Available from <https://doi.org/10.1021/acs.biomac.5b00188>.
- Mauro, A. (1961). Satellite cell of skeletal muscle fibers. *The Journal of Biophysical and Biochemical Cytology*, 9, 493–495. Available from <https://doi.org/10.1083/jcb.9.2.493>.
- McBeth, C., Lauer, J., Ottersbach, M., Campbell, J., Sharon, A., & Sauer-Budge, A. F. (2017). 3D bioprinting of GelMA scaffolds triggers mineral deposition by primary human osteoblasts. *Biofabrication*, 9, 15009. Available from <https://doi.org/10.1088/1758-5090/aa53bd>.
- McCain, M. L., Agarwal, A., Nesmith, H. W., Nesmith, A. P., & Parker, K. K. (2014). Micromolded gelatin hydrogels for extended culture of engineered cardiac tissues. *Biomaterials*, 35, 5462–5471. Available from <https://doi.org/10.1016/j.biomaterials.2014.03.052>.
- McElheny, C., Hayes, D., & Devireddy, R. (2017). Design and fabrication of a low-cost three-dimensional bioprinter. *Journal of Medical Devices*, 11, 41001. Available from <https://doi.org/10.1115/1.4037259>.
- McMillin, D. W., Delmore, J., Weisberg, E., Negri, J. M., Geer, D. C., Klippel, S., . . . Mitsiades, C. S. (2010). Tumor cell-specific bioluminescence platform to identify stroma-induced changes to anticancer drug activity. *Nature Medicine*, 16, 483–489. Available from <https://doi.org/10.1038/nm.2112>.
- McMillin, D. W., Negri, J. M., & Mitsiades, C. S. (2013). The role of tumour–stromal interactions in modifying drug response: Challenges and opportunities. *Nature Reviews: Drug Discovery*, 12, 217–228. Available from <https://doi.org/10.1038/nrd3870>.
- Medvedev, S. P., Shevchenko, A. I., & Zakian, S. M. (2010). Induced pluripotent stem cells: Problems and advantages when applying them in regenerative medicine. *Acta Naturae*, 2, 18–28.
- Merceron, T. K., Burt, M., Seol, Y.-J., Kang, H.-W., Lee, S. J., Yoo, J. J., & Atala, A. (2015). A 3D bioprinted complex structure for engineering the muscle–tendon unit. *Biofabrication*, 7, 035003. Available from <https://doi.org/10.1088/1758-5090/7/3/035003>.
- Miao, S., Zhu, W., Castro, N. J., Nowicki, M., Zhou, X., Cui, H., . . . Zhang, L. G. (2016). 4D printing smart biomedical scaffolds with novel soybean oil epoxidized acrylate. *Scientific Reports*, 6, 27226. Available from <https://doi.org/10.1038/srep27226>.

- Miao, T., Miller, E. J., McKenzie, C., & Oldinski, R. A. (2015). Physically crosslinked polyvinyl alcohol and gelatin interpenetrating polymer network theta-gels for cartilage regeneration. *Journal of Materials Chemistry B*, 3, 9242–9249. Available from <https://doi.org/10.1039/C5TB00989H>.
- Mistry, P., Aied, A., Alexander, M., Shakesheff, K., Bennett, A., & Yang, J. (2017). Bioprinting using mechanically robust core-shell cell-laden hydrogel strands. *Macromolecular Bioscience*, 17, 1600472. Available from <https://doi.org/10.1002/mabi.201600472>.
- Morris, V. B., Nimbalkar, S., Younesi, M., McClellan, P., & Akkus, O. (2017). Mechanical properties, cytocompatibility and manufacturability of chitosan: PEGDA hybrid-gel scaffolds by stereolithography. *Annals of Biomedical Engineering*, 45, 286–296. Available from <https://doi.org/10.1007/s10439-016-1643-1>.
- Morrison, G., Liu, C., Wing, C., Delaney, S. M., Zhang, W., & Dolan, M. E. (2016). Evaluation of inter-batch differences in stem-cell derived neurons. *Stem Cell Research*, 16, 140–148. Available from <https://doi.org/10.1016/j.scr.2015.12.025>.
- Mozaffarian, D., Benjamin, E. J., Go, A. S., Arnett, D. K., Blaha, M. J., Cushman, M., . . . American Heart Association Statistics Committee and Stroke Statistics Subcommittee. (2015). Heart disease and stroke statistics—2015 update: A report from the American Heart Association. *Circulation*, 131. Available from <https://doi.org/10.1161/CIR.000000000000152>, e29–322.
- Murphy, S. V., & Atala, A. (2014). 3D bioprinting of tissues and organs. *Nature Biotechnology*, 32, 773–785. Available from <https://doi.org/10.1038/nbt.2958>.
- Müller, M., Öztürk, E., Arlov, Ø., Gatenholm, P., & Zenobi-Wong, M. (2017). Alginate sulfate–nanocellulose bioinks for cartilage bioprinting applications. *Annals of Biomedical Engineering*, 45, 210–223. Available from <https://doi.org/10.1007/s10439-016-1704-5>.
- O’Connell, C. D., Di Bella, C., Thompson, F., Augustine, C., Beirne, S., Cornock, R., . . . Wallace, G. G. (2016). Development of the biopen: A handheld device for surgical printing of adipose stem cells at a chondral wound site. *Biofabrication*, 8, 15019. Available from <https://doi.org/10.1088/1758-5090/8/1/015019>.
- Ostrom, Q. T., Cioffi, G., Gittleman, H., Patil, N., Waite, K., Kruchko, C., & Barnholtz-Sloan, J. S. (2019). CBTRUS statistical report: Primary brain and other central nervous system tumors diagnosed in the United States in 2012–2016. *Neuro-Oncology*, 21, v1–v100. Available from <https://doi.org/10.1093/neuonc/noz150>.
- Ouyang, L., Yao, R., Mao, S., Chen, X., Na, J., & Sun, W. (2015). Three-dimensional bioprinting of embryonic stem cells directs highly uniform embryoid body formation. *Biofabrication*, 7, 44101. Available from <https://doi.org/10.1088/1758-5090/7/4/044101>.
- Ouyang, L., Yao, R., Zhao, Y., & Sun, W. (2016). Effect of bioink properties on printability and cell viability for 3D bioplotting of embryonic stem cells. *Biofabrication*, 8, 35020. Available from <https://doi.org/10.1088/1758-5090/8/3/035020>.
- Ozbolat, I. T., & Hospodiuk, M. (2016). Current advances and future perspectives in extrusion-based bioprinting. *Biomaterials*, 76, 321–343. Available from <https://doi.org/10.1016/j.biomaterials.2015.10.076>.
- Pati, F., Jang, J., Ha, D.-H., Won Kim, S., Rhie, J.-W., Shim, J.-H., . . . Cho, D.-W. (2014). Printing three-dimensional tissue analogues with decellularized extracellular matrix bioink. *Nature Communications*, 5, 1–11. Available from <https://doi.org/10.1038/ncomms4935>.
- Paxton, N., Smolan, W., Böck, T., Melchels, F., Groll, J., & Jungst, T. (2017). Proposal to assess printability of bioinks for extrusion-based bioprinting and evaluation of rheological properties governing bioprintability. *Biofabrication*, 9, 44107. Available from <https://doi.org/10.1088/1758-5090/aa8dd8>.
- Peng, W., Datta, P., Ayan, B., Ozbolat, V., Sosnoski, D., & Ozbolat, I. T. (2017). 3D bioprinting for drug discovery and development in pharmaceuticals. *Acta Biomaterialia*, 57, 26–46. Available from <https://doi.org/10.1016/j.actbio.2017.05.025>.

- Pereira, R. F., & Bártolo, P. J. (2015). 3D bioprinting of photocrosslinkable hydrogel constructs. *Journal of Applied Polymer Science*, 132, 42458. Available from <https://doi.org/10.1002/app.42458>.
- Potyondy, T., Uquillas, J. A., Tebon, P. J., Byambaa, B., Hasan, A., Tavafoghi, M., ... Ashammakhi, N. (2020). Recent advances in 3D bioprinting of musculoskeletal tissues. *Biofabrication*. Available from <https://doi.org/10.1088/1758-5090/abc8de>.
- Rao, C., Prodromakis, T., Kolker, L., Chaudhry, U. A. R., Trantidou, T., Sridhar, A., ... Terracciano, C. M. (2013). The effect of microgrooved culture substrates on calcium cycling of cardiac myocytes derived from human induced pluripotent stem cells. *Biomaterials*, 34, 2399–2411. Available from <https://doi.org/10.1016/j.biomaterials.2012.11.055>.
- Regehly, M., Garmshausen, Y., Reuter, M., König, N. F., Israel, E., Kelly, D. P., ... Hecht, S. (2020). Xolography for linear volumetric 3D printing. *Nature*, 588, 620–624. Available from <https://doi.org/10.1038/s41586-020-3029-7>.
- Reid, J. A., Mollica, P. A., Johnson, G. D., Ogle, R. C., Bruno, R. D., & Sachs, P. C. (2016). Accessible bioprinting: Adaptation of a low-cost 3D-printer for precise cell placement and stem cell differentiation. *Biofabrication*, 8, 25017. Available from <https://doi.org/10.1088/1758-5090/8/2/025017>.
- Ribas, J., Sadeghi, H., Manbachi, A., Leijten, J., Brinegar, K., Zhang, Y. S., ... Khademhosseini, A. (2016). Cardiovascular organ-on-a-chip platforms for drug discovery and development. *Applied In Vitro Toxicology*, 2, 82–96. Available from <https://doi.org/10.1089/aivt.2016.0002>.
- Ribeiro, A., Blokzijl, M. M., Levato, R., Visser, C. W., Castilho, M., Hennink, W. E., ... Malda, J. (2017). Assessing bioink shape fidelity to aid material development in 3D bioprinting. *Biofabrication*, 10, 14102. Available from <https://doi.org/10.1088/1758-5090/aa90e2>.
- Ribeiro, A. J. S., Ang, Y.-S., Fu, J.-D., Rivas, R. N., Mohamed, T. M. A., Higgs, G. C., ... Pruitt, B. L. (2015). Contractility of single cardiomyocytes differentiated from pluripotent stem cells depends on physiological shape and substrate stiffness. *Proceedings of the National Academy of Sciences*, 112, 12705–12710. Available from <https://doi.org/10.1073/pnas.1508073112>.
- Rimann, M., Bono, E., Annaheim, H., Bleisch, M., & Graf-Hausner, U. (2016). Standardized 3D bioprinting of soft tissue models with human primary cells. *Journal of Laboratory Automation*, 21, 496–509. Available from <https://doi.org/10.1177/2211068214567146>.
- Rocca, M., Fragasso, A., Liu, W., Heinrich, M. A., & Zhang, Y. S. (2018). Embedded multimaterial extrusion bioprinting. *SLAS Technology: Translating Life Sciences Innovation*, 23, 154–163. Available from <https://doi.org/10.1177/2472630317742071>.
- Rodriguez, M. J., Dixon, T. A., Cohen, E., Huang, W., Omenetto, F. G., & Kaplan, D. L. (2018). 3D freeform printing of silk fibroin. *Acta Biomaterialia*, 71, 379–387. Available from <https://doi.org/10.1016/j.actbio.2018.02.035>.
- Russo, V., Yu, C., Belliveau, P., Hamilton, A., & Flynn, L. E. (2014). Comparison of human adipose-derived stem cells isolated from subcutaneous, omental, and intrathoracic adipose tissue depots for regenerative applications. *Stem Cells Translational Medicine*, 3, 206–217. Available from <https://doi.org/10.5966/sctm.2013-0125>.
- Rutz, A. L., Hyland, K. E., Jakus, A. E., Burghardt, W. R., & Shah, R. N. (2015). A multimaterial bioink method for 3D printing tunable, cell-compatible hydrogels. *Advanced Materials*, 27, 1607–1614. Available from <https://doi.org/10.1002/adma.201405076>.
- Sakai, S., Ohi, H., Hotta, T., Kamei, H., & Taya, M. (2018). Differentiation potential of human adipose stem cells bioprinted with hyaluronic acid/gelatin-based bioink through microextrusion and visible light-initiated crosslinking. *Biopolymers*, 109, e23080. Available from <https://doi.org/10.1002/bip.23080>.
- Sakai, S., Ueda, K., Gantumur, E., Taya, M., & Nakamura, M. (2018). Drop-on-drop multimaterial 3D bioprinting realized by peroxidase-mediated cross-linking. *Macromolecular Rapid Communications*, 39, 1700534. Available from <https://doi.org/10.1002/marc.201700534>.

- Sakai, S., Kamei, H., Mori, T., Hotta, T., Ohi, H., Nakahata, M., & Taya, M. (2018). Visible light-induced hydrogelation of an alginate derivative and application to stereolithographic bioprinting using a visible light projector and acid red. *Biomacromolecules*, 19, 672–679. Available from <https://doi.org/10.1021/acs.biomac.7b01827>.
- Salick, M. R., Napiwocki, B. N., Sha, J., Knight, G. T., Chindhy, S. A., Kamp, T. J., ... Crone, W. C. (2014). Micropattern width dependent sarcomere development in human ESC-derived cardiomyocytes. *Biomaterials*, 35, 4454–4464. Available from <https://doi.org/10.1016/j.biomaterials.2014.02.001>.
- Samson, A. A. S., Lee, J., & Song, J. M. (2018). Paper-based inkjet bioprinting to detect fluorescence resonance energy transfer for the assessment of anti-inflammatory activity. *Scientific Reports*, 8, 591. Available from <https://doi.org/10.1038/s41598-017-18995-3>.
- Sanz, B., Albillos Sanchez, A., Tangey, B., Gilmore, K., Yue, Z., Liu, X., & Wallace, G. (2020). Light cross-linkable marine collagen for coaxial printing of a 3D model of neuromuscular junction formation. *Biomedicines*, 9, 16. Available from <https://doi.org/10.3390/biomedicines9010016>.
- Scaglione, S., Kliethermes, S., Cao, G., Shoham, D., Durazo, R., Luke, A., & Volk, M. L. (2015). The epidemiology of cirrhosis in the United States: A population-based study. *Journal of Clinical Gastroenterology*, 49, 690–696. Available from <https://doi.org/10.1097/MCG.0000000000000208>.
- Schütz, K., Placht, A.-M., Paul, B., Brüggemeier, S., Gelinsky, M., & Lode, A. (2017). Three-dimensional plotting of a cell-laden alginate/methylcellulose blend: Towards biofabrication of tissue engineering constructs with clinically relevant dimensions. *Journal of Tissue Engineering and Regenerative Medicine*, 11, 1574–1587. Available from <https://doi.org/10.1002/term.2058>.
- Shafiee, A., McCune, M., Forgacs, G., & Kosztin, I. (2015). Post-deposition bioink self-assembly: A quantitative study. *Biofabrication*, 7, 45005. Available from <https://doi.org/10.1088/1758-5090/7/4/045005>.
- Shanjani, Y., Pan, C. C., Elomaa, L., & Yang, Y. (2015). A novel bioprinting method and system for forming hybrid tissue engineering constructs. *Biofabrication*, 7, 45008. Available from <https://doi.org/10.1088/1758-5090/7/4/045008>.
- Shao, H., Yang, X., He, Y., Fu, J., Liu, L., Ma, L., ... Gou, Z. (2015). Bioactive glass-reinforced bioceramic ink writing scaffolds: Sintering, microstructure and mechanical behavior. *Biofabrication*, 7, 35010. Available from <https://doi.org/10.1088/1758-5090/7/3/035010>.
- Singelyn, J. M., DeQuach, J. A., Seif-Naraghi, S. B., Littlefield, R. B., Schup-Magoffin, P. J., & Christman, K. L. (2009). Naturally derived myocardial matrix as an injectable scaffold for cardiac tissue engineering. *Biomaterials*, 30, 5409–5416. Available from <https://doi.org/10.1016/j.biomaterials.2009.06.045>.
- Sivaraman, L. G., Leach, A., Townsend, J. K., Iida, S., Hogan, T., Stolz, B. J., ... Griffith, S. R. (2005). A microscale *in vitro* physiological model of the liver: Predictive screens for drug metabolism and enzyme induction. *Current Drug Metabolism*, 6, 569–591. Available from <https://doi.org/10.2174/138920009787048400>.
- Skardal, A., & Atala, A. (2015). Biomaterials for integration with 3-D bioprinting. *Annals of Biomedical Engineering*, 43, 730–746. Available from <https://doi.org/10.1007/s10439-014-1207-1>.
- Skardal, A., Devarasetty, M., Kang, H. W., Mead, I., Bishop, C., Shupe, T., ... Atala, A. (2015). A hydrogel bioink toolkit for mimicking native tissue biochemical and mechanical properties in bioprinted tissue constructs. *Acta Biomaterialia*, 25, 24–34. Available from <https://doi.org/10.1016/j.actbio.2015.07.030>.
- Smith, C. M., Christian, J. J., Warren, W. L., & Williams, S. K. (2007). Characterizing environmental factors that impact the viability of tissue-engineered constructs fabricated by a direct-write bioassembly tool. *Tissue Engineering*, 13, 373–383. Available from <https://doi.org/10.1089/ten.2006.0101>.
- Soman, P., Fozdar, D. Y., Lee, J. W., Phadke, A., Varghese, S., & Chen, S. (2012). A three-dimensional polymer scaffolding material exhibiting a zero Poisson's ratio. *Soft Matter*, 8, 4946. Available from <https://doi.org/10.1039/c2sm07354d>.

- Soman, P., Tobe, B. T. D., Lee, J. W., Winquist, A. A. M., Singec, I., Vecchio, K. S., ... Chen, S. (2012). Three-dimensional scaffolding to investigate neuronal derivatives of human embryonic stem cells. *Biomedical Microdevices*, 14, 829–838. Available from <https://doi.org/10.1007/s10544-012-9662-7>.
- Stanton, M. M., Samitier, J., & Sánchez, S. (2015). Bioprinting of 3D hydrogels. *Lab on a Chip*, 15, 3111–3115. Available from <https://doi.org/10.1039/C5LC90069G>.
- Steele, A. N., MacArthur, J. W., & Woo, Y. J. (2017). Stem cell therapy: Healing or hype? Why stem cell delivery doesn't work. *Circulation Research*, 120, 1868–1870. Available from <https://doi.org/10.1161/CIRCRESAHA.117.310584>.
- Stichler, S., Böck, T., Paxton, N., Bertlein, S., Levato, R., Schill, V., ... Groll, J. (2017). Double printing of hyaluronic acid/poly(glycidol) hybrid hydrogels with poly(ϵ -caprolactone) for MSC chondrogenesis. *Biofabrication*, 9, 44108. Available from <https://doi.org/10.1088/1758-5090/aa8cb7>.
- Stichler, S., Jungst, T., Schamel, M., Zilkowski, I., Kuhlmann, M., Böck, T., ... Groll, J. (2017). Thiol-ene clickable poly(glycidol) hydrogels for biofabrication. *Annals of Biomedical Engineering*, 45, 273–285. Available from <https://doi.org/10.1007/s10439-016-1633-3>.
- Su, P. J., Tran, Q. A., Fong, J. J., Eliceiri, K. W., Ogle, B. M., & Campagnola, P. J. (2012). Mesenchymal stem cell interactions with 3D ECM modules fabricated via multiphoton excited photochemistry. *Biomacromolecules*, 13, 2917–2925. Available from <https://doi.org/10.1021/bm300949k>.
- Tabriz, A. G., Hermida, M. A., Leslie, N. R., & Shu, W. (2015). Three-dimensional bioprinting of complex cell laden alginate hydrogel structures. *Biofabrication*, 7, 45012. Available from <https://doi.org/10.1088/1758-5090/7/4/045012>.
- Takayama, K., Kawabata, K., Nagamoto, Y., Kishimoto, K., Tashiro, K., Sakurai, F., ... Mizuguchi, H. (2013). 3D spheroid culture of hESC/hiPSC-derived hepatocyte-like cells for drug toxicity testing. *Biomaterials*, 34, 1781–1789. Available from <https://doi.org/10.1016/j.biomaterials.2012.11.029>.
- Tan, Y. J., Tan, X., Yeong, W. Y., & Tor, S. B. (2016). Hybrid microsc scaffold-based 3D bioprinting of multi-cellular constructs with high compressive strength: A new biofabrication strategy. *Scientific Reports*, 6, 39140. Available from <https://doi.org/10.1038/srep39140>.
- Tang, M., Xie, Q., Gimple, R. C., Zhong, Z., Tam, T., Tian, J., ... Rich, J. N. (2020). Three-dimensional bioprinted glioblastoma microenvironments model cellular dependencies and immune interactions. *Cell Research*, 1–21. Available from <https://doi.org/10.1038/s41422-020-0338-1>.
- Tang, M., Tiwari, S. K., Agrawal, K., Tan, M., Dang, J., Tam, T., ... Chen, S. (2021). Rapid 3D bioprinting of glioblastoma model mimicking native biophysical heterogeneity. *Small (Weinheim an der Bergstrasse, Germany)*, 2006050. Available from <https://doi.org/10.1002/sml.202006050>.
- Tao, J., Hu, Y., Wang, S., Zhang, J., Liu, X., Gou, Z., ... Gou, M. (2017). A 3D-engineered porous conduit for peripheral nerve repair. *Scientific Reports*, 7, 46038. Available from <https://doi.org/10.1038/srep46038>.
- Tijore, A., Behr, J.-M., Irvine, S. A., Baisane, V., & Venkatraman, S. (2018). Bioprinted gelatin hydrogel platform promotes smooth muscle cell contractile phenotype maintenance. *Biomedical Microdevices*, 20, 32. Available from <https://doi.org/10.1007/s10544-018-0274-8>.
- Toh, Y.-C., Lim, T. C., Tai, D., Xiao, G., van Noort, D., & Yu, H. (2009). A microfluidic 3D hepatocyte chip for drug toxicity testing. *Lab on a Chip*, 9, 2026–2035. Available from <https://doi.org/10.1039/b900912d>.
- Truby, R. L., & Lewis, J. A. (2016). Printing soft matter in three dimensions. *Nature*, 540, 371–378. Available from <https://doi.org/10.1038/nature21003>.
- Tsang, V. L., Chen, A. A., Cho, L. M., Jadin, K. D., Sah, R. L., DeLong, S., ... Bhatia, S. N. (2007). Fabrication of 3D hepatic tissues by additive photopatterning of cellular hydrogels. *FASEB Journal*, 21, 790–801. Available from <https://doi.org/10.1096/fj.06-7117com>.
- Tse, C. C. W., & Smith, P. J. (2018). Inkjet printing for biomedical applications. *Methods in Molecular Biology*, 1771, 107–117. Available from https://doi.org/10.1007/978-1-4939-7792-5_9.

- Unger, C., Kramer, N., Walzl, A., Scherzer, M., Hengstschläger, M., & Dolznig, H. (2014). Modeling human carcinomas: Physiologically relevant 3D models to improve anti-cancer drug development. *Advanced Drug Delivery Reviews*, 79, 50–67. Available from <https://doi.org/10.1016/j.addr.2014.10.015>.
- Vinson, B. T., Phamduy, T. B., Shipman, J., Riggs, B., Strong, A. L., Sklare, S. C., ... Chrisey, D. B. (2017). Laser direct-write based fabrication of a spatially-defined, biomimetic construct as a potential model for breast cancer cell invasion into adipose tissue. *Biofabrication*, 9, 25013. Available from <https://doi.org/10.1088/1758-5090/aa6bad>.
- Wang, L., Xu, M., Luo, L., Zhou, Y., & Si, P. (2018). Iterative feedback bio-printing-derived cell-laden hydrogel scaffolds with optimal geometrical fidelity and cellular controllability. *Scientific Reports*, 8, 2802. Available from <https://doi.org/10.1038/s41598-018-21274-4>.
- Wang, L. L., Highley, C. B., Yeh, Y.-C., Galarraga, J. H., Uman, S., & Burdick, J. A. (2018). Three-dimensional extrusion bioprinting of single- and double-network hydrogels containing dynamic covalent crosslinks. *Journal of Biomedical Materials Research Part A*, 106, 865–875. Available from <https://doi.org/10.1002/jbm.a.36323>.
- Wang, P., Berry, D., Moran, A., He, F., Tam, T., Chen, L., & Chen, S. (2019). Controlled growth factor release in 3D-printed hydrogels. *Advanced Healthcare Materials*, 1900977. Available from <https://doi.org/10.1002/adhm.201900977>.
- Wang, P., Berry, D. B., Song, Z., Kiratitanaporn, W., Schimelman, J., Moran, A., ... Chen, S. (2020). 3D Printing of a biocompatible double network elastomer with digital control of mechanical properties. *Advanced Functional Materials*, 30, 1910391. Available from <https://doi.org/10.1002/adfm.201910391>.
- Wang, Z., Abdulla, R., Parker, B., Samanipour, R., Ghosh, S., & Kim, K. (2015). A simple and high-resolution stereolithography-based 3D bioprinting system using visible light crosslinkable bioinks. *Biofabrication*, 7, 45009. Available from <https://doi.org/10.1088/1758-5090/7/4/045009>.
- Wang, Z., Tian, Z., Jin, X., Holzman, J. F., Menard, F., & Kim, K. (2017). *Visible light-based stereolithography bioprinting of cell-adhesive gelatin hydrogels*. 2017 39th annual international conference of the IEEE Engineering in Medicine and Biology Society (EMBC) (pp. 1599–1602). IEEE. Available from <https://doi.org/10.1109/EMBC.2017.8037144>.
- Wang, Z., Jin, X., Tian, Z., Menard, F., Holzman, J. F., & Kim, K. (2018). A novel, well-resolved direct laser bioprinting system for rapid cell encapsulation and microwell fabrication. *Advanced Healthcare Materials*, 1701249. Available from <https://doi.org/10.1002/adhm.201701249>.
- Warner, J., Soman, P., Zhu, W., Tom, M., & Chen, S. (2016). Design and 3D printing of hydrogel scaffolds with fractal geometries. *ACS Biomaterials Science and Engineering*, 2, 1763–1770. Available from <https://doi.org/10.1021/acsbiomaterials.6b00140>.
- Wu, Z., Su, X., Xu, Y., Kong, B., Sun, W., & Mi, S. (2016). Bioprinting three-dimensional cell-laden tissue constructs with controllable degradation. *Scientific Reports*, 6, 24474. Available from <https://doi.org/10.1038/srep24474>.
- Wylie, R. G., Ahsan, S., Aizawa, Y., Maxwell, K. L., Morshead, C. M., & Shoichet, M. S. (2011). Spatially controlled simultaneous patterning of multiple growth factors in three-dimensional hydrogels. *Nature Materials*, 10, 799–806. Available from <https://doi.org/10.1038/nmat3101>.
- Xiong, R., Zhang, Z., Chai, W., Huang, Y., & Chrisey, D. B. (2015). Freeform drop-on-demand laser printing of 3D alginate and cellular constructs. *Biofabrication*, 7, 45011. Available from <https://doi.org/10.1088/1758-5090/7/4/045011>.
- Xiong, R., Zhang, Z., Chai, W., Chrisey, D. B., & Huang, Y. (2017). Study of gelatin as an effective energy absorbing layer for laser bioprinting. *Biofabrication*, 9, 24103. Available from <https://doi.org/10.1088/1758-5090/aa74f2>.

- Xu, C., Lee, W., Dai, G., & Hong, Y. (2018). Highly elastic biodegradable single-network hydrogel for cell printing. *ACS Applied Materials & Interfaces*, 10, 9969–9979. Available from <https://doi.org/10.1021/acsami.8b01294>.
- Xu, F., Celli, J., Rizvi, I., Moon, S., Hasan, T., & Demirci, U. (2011). A three-dimensional *in vitro* ovarian cancer coculture model using a high-throughput cell patterning platform. *Biotechnology Journal*, 6, 204–212. Available from <https://doi.org/10.1002/biot.201000340>.
- Yang, W., Yu, H., Wei, F., Li, G., Wang, Y., & Liu, L. (2015). Selective pattern of cancer cell accumulation and growth using UV modulating printing of hydrogels. *Biomedical Microdevices*, 17, 104. Available from <https://doi.org/10.1007/s10544-015-0013-3>.
- Yang, Y., Du, T., Zhang, J., Kang, T., Luo, L., Tao, J., ... Gou, M. (2017). A 3D-engineered conformal implant releases DNA nanocomplexes for eradicating the postsurgery residual glioblastoma. *Advanced Science*, 4. Available from <https://doi.org/10.1002/advs.201600491>.
- Yao, T., & Asayama, Y. (2017). Animal-cell culture media: History, characteristics, and current issues. *Reproductive Medicine and Biology*, 16, 99–117. Available from <https://doi.org/10.1002/rmb2.12024>.
- Ye, K. Y., Sullivan, K. E., & Black, L. D. (2011). Encapsulation of cardiomyocytes in a fibrin hydrogel for cardiac tissue engineering. *Journal of Visualized Experiments*, 3251. Available from <https://doi.org/10.3791/3251>.
- Yin, J., Yan, M., Wang, Y., Fu, J., & Suo, H. (2018). 3D bioprinting of low-concentration cell-laden gelatin methacrylate (GelMA) bioinks with a two-step cross-linking strategy. *ACS Applied Materials & Interfaces*, 10, 6849–6857. Available from <https://doi.org/10.1021/acsami.7b16059>.
- Yoon, H., Lee, J.-S., Yim, H., Kim, G., & Chun, W. (2016). Development of cell-laden 3D scaffolds for efficient engineered skin substitutes by collagen gelation. *RSC Advances*, 6, 21439–21447. Available from <https://doi.org/10.1039/C5RA19532B>.
- Yoon No, D., Lee, K.-H., Lee, J., & Lee, S.-H. (2015). 3D liver models on a microplatform: Well-defined culture, engineering of liver tissue and liver-on-a-chip. *Lab on a Chip*, 15, 3822–3837. Available from <https://doi.org/10.1039/C5LC00611B>.
- You, F., Eames, B. F., & Chen, X. (2017). Application of extrusion-based hydrogel bioprinting for cartilage tissue engineering. *International Journal of Molecular Sciences*, 18, 1597. Available from <https://doi.org/10.3390/ijms18071597>.
- Yu, C., Ma, X., Zhu, W., Wang, P., Miller, K. L., Stupin, J., ... Chen, S. (2019). Scanningless and continuous 3D bioprinting of human tissues with decellularized extracellular matrix. *Biomaterials*, 194, 1–13. Available from <https://doi.org/10.1016/j.biomaterials.2018.12.009>.
- Zhang, W., Soman, P., Meggs, K., Qu, X., & Chen, S. (2013). Tuning the Poisson's ratio of biomaterials for investigating cellular response. *Advanced Functional Materials*, 23, 3226–3232. Available from <https://doi.org/10.1002/adfm.201202666>.
- Zhang, Y. S., Arneri, A., Bersini, S., Shin, S. R., Zhu, K., Goli-Malekabadi, Z., ... Khademhosseini, A. (2016). Bioprinting 3D microfibrillar scaffolds for engineering endothelialized myocardium and heart-on-a-chip. *Biomaterials*, 110, 45–59. Available from <https://doi.org/10.1016/j.biomaterials.2016.09.003>.
- Zhang, Z., Xiong, R., Mei, R., Huang, Y., & Chrisey, D. B. (2015). Time-resolved imaging study of jetting dynamics during laser printing of viscoelastic alginate solutions. *Langmuir: The ACS Journal of Surfaces and Colloids*, 31, 6447–6456. Available from <https://doi.org/10.1021/acs.langmuir.5b00919>.
- Zhang, Z., Xu, C., Xiong, R., Chrisey, D. B., & Huang, Y. (2017). Effects of living cells on the bioink printability during laser printing. *Biomicrofluidics*, 11, 34120. Available from <https://doi.org/10.1063/1.4985652>.
- Zhang, Z., Chai, W., Xiong, R., Zhou, L., & Huang, Y. (2017). Printing-induced cell injury evaluation during laser printing of 3T3 mouse fibroblasts. *Biofabrication*, 9, 25038. Available from <https://doi.org/10.1088/1758-5090/aa6ed9>.

- Zhao, Y., Yao, R., Ouyang, L., Ding, H., Zhang, T., Zhang, K., . . . Sun, W. (2014). Three-dimensional printing of Hela cells for cervical tumor model *in vitro*. *Biofabrication*, 6, 35001. Available from <https://doi.org/10.1088/1758-5082/6/3/035001>.
- Zhong, Z., Deng, X., Wang, P., Yu, C., Kiratitanaporn, W., Wu, X., . . . Chen, S. (2021). Rapid bioprinting of conjunctival stem cell micro-constructs for subconjunctival ocular injection. *Biomaterials*, 267, 120462. Available from <https://doi.org/10.1016/j.biomaterials.2020.120462>.
- Zhu, J. (2010). Bioactive modification of poly(ethylene glycol) hydrogels for tissue engineering. *Biomaterials*, 31, 4639–4656. Available from <https://doi.org/10.1016/j.biomaterials.2010.02.044>.
- Zhu, W., Li, J., Leong, Y. J., Rozen, I., Qu, X., Dong, R., . . . Chen, S. (2015). 3D-printed artificial microfish. *Advanced Materials*, 27, 4411–4417. Available from <https://doi.org/10.1002/adma.201501372>.
- Zhu, W., Ma, X., Gou, M., Mei, D., Zhang, K., & Chen, S. (2016). 3D printing of functional biomaterials for tissue engineering. *Current Opinion in Biotechnology*, 40, 103–112. Available from <https://doi.org/10.1016/j.copbio.2016.03.014>.
- Zhu, W., Qu, X., Zhu, J., Ma, X., Patel, S., Liu, J., . . . Chen, S. (2017). Direct 3D bioprinting of prevascularized tissue constructs with complex microarchitecture. *Biomaterials*, 124, 106–115. Available from <https://doi.org/10.1016/j.biomaterials.2017.01.042>.
- Zipfel, W. R., Williams, R. M., & Webb, W. W. (2003). Nonlinear magic: Multiphoton microscopy in the biosciences. *Nature Biotechnology*, 21, 1369–1377. Available from <https://doi.org/10.1038/nbt899>.

3D BIOPRINTING TECHNIQUES

3

Binil Starly and Rohan Shirwaiker*Department of Industrial and Systems Engineering, North Carolina State University, Raleigh, NC, United States***3.1 INTRODUCTION**

Critical to the success of tissue engineering and regenerative medicine (TERM) approaches, which require the use of a structural matrix, is the design of the scaffold and ensuing tissue construct. Cells involved in the regeneration process are influenced by the macro- and microarchitecture of the constructs. Two primary approaches have been developed to produce scaffolds and tissue constructs: (1) chemically driven processes, such as gas foaming, solvent casting, salt leaching, and freeze casting; and (2) computer-aided layered manufacturing-based approaches. Scaffolds produced through either of these approaches create structural matrices with defined pore architecture in terms of size, shape, and orientation. The three-dimensional (3D) surface area offered by these scaffolds serves as anchoring surfaces for cell adhesion, proliferation, and differentiation to the desired tissue type and function. In these approaches, scaffolds are produced first and the cells of interest are added in a subsequent processing step. The main drawbacks of this approach are the lack of cellular penetration and highly variable cell distribution within the scaffold matrix, primarily seen in scaffold sizes larger than 5 mm in thickness. These limitations arise from two reasons: (1) low cell seeding efficiency at the initial stages to fully inoculate the scaffold itself; and (2) lack of cellular proliferation deep into the scaffold architecture primarily due to rapid drop-off of nutrient concentration within the scaffold core. Several approaches have been developed by the research community over the last decade to mitigate these disadvantages. One popular approach has been the adoption of perfusion-based bioreactor systems to help improve cell seeding efficiency and the transport of nutrients within the scaffold to promote more uniform cellular adhesion and proliferation. An added benefit is that these bioreactors can be customized to provide mechanical and chemical stimulation to accelerate tissue formation. While generally successful, these methods are often limited to small-size defects and thus cannot be translated to thicker tissue constructs.

A more exciting approach is to directly involve cells within the construct design and fabrication process. This offers the advantage of combining multiple processing steps together through which a cellular construct is directly achieved. This approach helps to overcome the seeding efficiency and cell distribution problem. It opens up newer opportunities to customize and regulate the cellular microenvironment by the controlled placement of cells and other biological molecules in defined spatial orientation. In recent years, this approach has rapidly taken off, with several fabrication processes being developed to build *in situ* cellular constructs. These processes include photopolymerization-based processes, laser-based patterning, contact stamping, and cellular microencapsulation to form multicellular tissue spheroids

and casting-based biocompatible processes to help achieve complex micro- and macroarchitecture (Valerie Liu Tsang, 2004; Derby, 2012). Perhaps one of the more exciting approaches is “3D bioprinting”-based methods due to the advantages they offer over competing methods to fabricate tissue and organ constructs. Among the advantages of this approach are

- (1) Capability to build both 2D and 3D structures
- (2) Ability to directly incorporate two or more different cell types in a defined spatial architecture in multi-scale patterns
- (3) Flexibility to achieve hybrid processes simply by switching out “tool” options as seen in conventional manufacturing machines, such as CNC-based systems. This suggests that multiple nozzle types or print heads can be incorporated to achieve heterogeneous structures.
- (4) Digitally enabled processes allow faster clinical translation and eventual approval by regulatory agencies. The control systems accompanying these processes offer the ability to control process parameters to help achieve desired cellular construct characteristics.

3.2 DEFINITION AND PRINCIPLES OF 3D BIOPRINTING

3D bioprinting is the process of automated deposition of biological molecules on a substrate to form a 3D heterogeneous functional structure with data derived from a digital model. The “print” material used in bioprinting techniques, also known as bioinks, often include a judicious combination of living biological cells, polymers, chemical factors, and biomolecules to form a physical and functional 3D living structure. The substrate is typically planar solid surfaces such as those of Petri dishes, glass slides, or wells of culture plates, although the concept can be extended to nonplanar, nonsolid, and flexible substrates. Living biological cells can be mammalian, insect, and plant-based as well as viruses and bacteria. 3D bioprinting has its roots in the conventional “ink-jet” process developed in the early 1950s that reproduces text and images from a computer file through droplets of ink deposited on a substrate such as paper. Much of the 3D bioprinting techniques have also grown from conventional additive manufacturing (AM) or layered manufacturing (LM) approaches. The complexity of the 3D bioprinting techniques, when compared to AM-based methods of scaffold fabrication, is attributed to the direct involvement of biological living materials during the fabrication process.

Hod Lipson in his book “Fabricated: The New World of 3D Printing” highlights 10 principles of 3D printing as guiding beacons to disrupt the current notion of manufacturing by reducing key barriers of time, cost, and skill level (Lipson, 2013). We have highlighted 10 principles of 3D bioprinting that will help shape the future of printing living tissue and organs for applications in regenerative medicine and tissue engineering.

Principle 1: Physical replication of the living construct from a digital blueprint file.

Bioprinting machines receive initial manufacturing process data input from a digital model of the construct. This digital model must serve as a repository of information that informs upstream and drives downstream manufacturing process activities.

Principle 2: Product customization with high degree of feature variety

Decision-makers and end-users of the printed construct must have the freedom to specify feature sets and functionalities of the construct with minimal complexity in manufacturing process activities.

Principle 3: Structurally heterogeneous product spanning more than two dimensional scales

Bioprinting processes must enable the realization of constructs with structural properties that vary in more than two dimensions. This is necessary to mimic the complexity of nature's own tissue and organ architecture. Processes must take advantage of repeating functional units often seen in complex organs, such as the liver and kidney.

Principle 4: Precise spatial patterning of "bio-ink" materials

The drop-on-demand and the continuous print capability combined with robotic automation in bioprinting processes enable the precise spatial patterning of biological entities in two and three dimensions.

Principle 5: Minimal handling and manipulation of living cells

All bioprinting processes will need to ensure minimal mechanical and environmental stress on nonliving biological molecules, chemical factors, and living biological cells. This is to ensure protein and growth factor stability and maximum cellular viability during fabrication.

Principle 6: Conductive microenvironment for functional cells before and after printing

Processes must provide a suitable microenvironment for cells to sustain themselves both prior to and post-printing. Both viability and functionality of cells must be minimally altered during the printing process.

Principle 7: Construct must be functionally stable for downstream operations.

Any printed construct that involves biomaterials and cells must be mechanically, chemically, and/or biologically stable for use in downstream applications. Structurally weak constructs cannot be handled by either humans or automated equipment. Chemically unstable structures will lead to disintegration of properties, while biologically unstable constructs simply cannot be used reliably for downstream processes.

Principle 8: Plug-n-Play bioprinting machines with minimal operator assistance

The machines should be relatively easy to operate with minimal input from operators and should be capable of being installed at any qualified manufacturing facility or hospital. This capability is essential to economically produce the final construct at a price acceptable to the end user. If the machines require very high skilled operation with labor-intensive monitoring, manufacturing costs will be untenable.

Principle 9: Cellular construct, engineered tissue and organs on demand

The digitally enabled technology provides the capability for decision-makers and end-users to custom specify features of the product and request them on-demand, reducing the need for large inventory stock-up of biomaterials and final product storage solutions.

Principle 10: Repeatable and assured functional quality of the bioprinted structure

Bioprinting processes must be accurate to initial design specifications, precise in terms of structural/ biological variability, and repeatable in terms of its operation. This is an essential requirement for any process to be qualified by regulatory agencies for any targeted biomedical application.

Several bioprinting processes have achieved varying degrees of adherence to each of the 10 listed principles. The processes themselves are continually being improved by several research groups through expanded biomaterial selection, improved accuracy of spatial arrangement, degree of automation, and complexity of structures generated. Processes successfully meeting all 10 principles will yield commercially viable fabrication processes for the scale-up manufacturing of engineered tissue and organoid systems. If developed well, processes will also find limited challenges to being integrated into any upstream operation that would define the entire manufacturing process cycle for TERM. In the following sections, we will go through some of the most widely investigated 3D bioprinting processes, their fundamental working principles, their application toward the fabrication of cellular constructs, and differences between each process.

3.3 3D BIOPRINTING TECHNOLOGIES

Scaffold-based regenerative medicine therapies involve the fabrication of scaffolds followed by seeding them with cells, preconditioning them inside an incubator, and then conditioning the construct inside a bioreactor to achieve adequate cell proliferation and function. Traditionally, both scaffold fabrication and cell seeding are two mutually separate and distinct steps in the process cycle. 3D Bioprinting techniques essentially combine both the scaffold fabrication and cell placement steps in an *in situ* layered manufacturing process step. The layer by layer bioprinting process enables the direct realization of scaffolding biomaterials, chemical molecules, and living cells in a desired spatial pattern to form the heterogeneous 3D construct. This feature was previously impossible to achieve with any of the conventionally produced tissue scaffolds or even with biopatterning approaches such as UV photopolymerization. The fundamental ability to “bioprint” essentially means that it is possible to accurately control the amount of “bioink” ejected out of the printhead or delivered on to a substrate. Figure 3.1 gives the generic architecture for a bioprinting machine with information and material flow paths highlighted among the entities contained with any system.

3D Bioprinting processes begin with a digital model definition of the cellular and tissue construct architecture to be fabricated. In the case of 2D patterns, the digital files can be directly coded into the

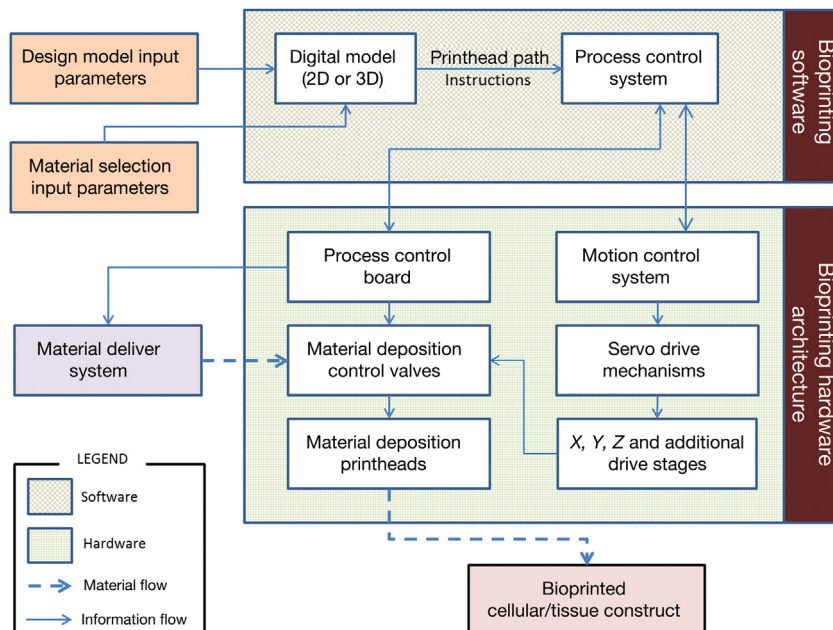


FIGURE 3.1

General 3D bioprinting hardware and software information and material flow.

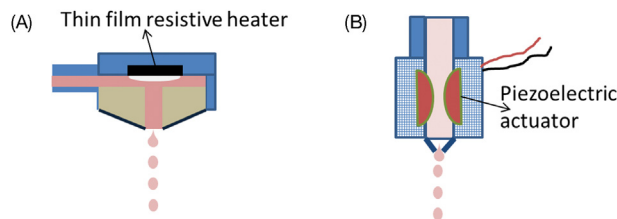
process control interface to drive the motion of printheads to help physically reproduce the architecture. This digital modeling of a complex 3D construct is typically performed in a computer-aided design (CAD) software environment. Both the external and internal architecture for the 3D construct can be designed with initial data obtained from CT/MRI images of the patient in need of a tissue replacement strategy. Image-based 3D reconstruction procedures can be carried out to help define the 3D digital model of the tissue replacement construct. Tools available in the software environment can help identify different material regions which specify the placement of the biomaterial matrix, biological molecules, and living cells. Process algorithms are written to convert the digital model to printhead path instructions necessary to drive the hardware systems. The exact format for machine instructions in such computer-aided manufacturing (CAM) depends on the printing technique and hardware configuration utilized. Once the signal for printing is activated, the process control system drives the bioprinting system hardware components for the physical realization of the printed construct.

Complex engineered tissue and cellular construct-based products will be made from spatially patterned cellular layers which ultimately will become large aggregates for a specialized tissue function. The entire operation must take place in sterile conditions to limit contamination of both source raw material and the final construct. If cells are involved in the fabrication process, the total time needed to produce a construct can be critical. The amount of time available is dependent on the cell type used. Unless printing conditions are well suited for cell maintenance, time to fabricate constructs should not exceed an hour. Longer times will result in reduced cellular viability and abnormally higher cellular stress, which will lead to degraded function.

We describe the main bioprinting techniques used by the research community to print biomaterials including cells and biomolecules to form 3D constructs. These 3D constructs can be used as tissue models for drug screening, as disease models to study cancer, and as constructs meant for animal or human implantation. Due to the size scale of cells, which are generally in the 5–20 μm size range, all bioprinting processes work at dimensional scale levels larger than the cell type utilized.

3.3.1 INK-JET-BASED BIOPRINTING

Ink-jet bioprinting is a noncontact printing process involving the precise deposition of picoliter to nanoliter droplets of “bioink” (a low-viscosity suspension of living cells, biomolecules, growth factors, etc.) onto a “biopaper” (a hydrogel substrate, culture dish, etc.) in a digitally controlled pattern. It is a direct adaptation of the conventional ink-jet printing process, and a majority of current ink-jet bioprinting activities continue to be conducted using partially modified commercially available desktop ink-jet printers. There are two fundamental approaches to ink-jet printing: continuous (CIJ) and drop-on-demand (DOD). In the CIJ approach, an uninterrupted stream of droplets is produced by forcing the ink through a microscopic nozzle orifice under pressure and deflecting it onto the substrate using an electrostatic field. Where droplet deposition is not required in the digital pattern, the droplets are steered into a gutter and collected for reuse. In the DOD approach, the ink droplets are ejected through the nozzle orifice by creating a pressure pulse inside a microfluidic chamber only when required. The DOD approach is of primary interest in bioprinting due to the pulsed nature of printing. The CIJ approach is not well suited to bioprinting due to the need for conductive ink formulations and the risk of contamination due to ink recirculation, among other reasons.

**FIGURE 3.2**

Inkjet printing mechanisms. (A) Thin film resistive heater generating a vapor bubble that ejects the bio-ink material; (B) A piezoelectric driven actuator that squeezes out a defined quantity of the bio-ink upon pulsed activation. Both mechanisms can either work in continuous or drop-on-demand jetting mode.

The DOD approach can be further categorized into thermal (heat) or piezoelectric (mechanical compression) based on the droplet actuation mechanism. A schematic of DOD ink-jet printing based on both mechanisms is presented in Figure 3.2. In thermal DOD, an electric current pulse applied to the heating element (thin film resistor) rapidly vaporizes a small pocket of ink in the microfluidic chamber. The resulting vapor bubble creates the pressure pulse that propels the ink droplet through the nozzle orifice and onto the substrate. In piezoelectric DOD, a microfluidic chamber above the nozzle contains a piezoelectric transducer for droplet actuation instead of a heating element. A voltage pulse applied to the transducer causes it to expand, creating the transient pressure that results in droplet ejection. For both forms of DOD, the rheological and surface tension properties of the ink govern their ability to be printed. The ink viscosity requirements vary from system to system, but a typical threshold is around 30 mPa/s (Reis et al., 2005; Seerden et al., 2001; Derby, 2008). In addition to the ink characteristics, the orifice size, the distance between nozzle and substrate, the frequency of the current pulse and resulting temperature gradient (thermal DOD), and the frequency of the voltage pulse and piezo-deformation characteristics of the transducer (piezoelectric DOD) have an effect on the ejected droplet size and spatial resolution in ink-jet printing.

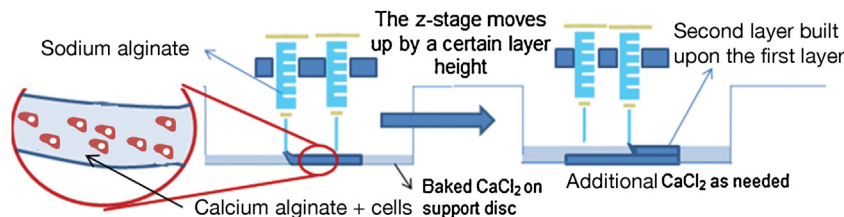
In addition to creating structures of nonliving biomolecules, such as DNA (Okamoto et al., 2000) and proteins (Delaney et al., 2009), DOD ink-jet bioprinters have been successfully used to print and pattern live mammalian cells, opening up new and exciting avenues in the field of tissue engineering and regenerative medicine. Ink-jet printing also offers the capability to print multiple cell types, biomaterials, or their combinations from different printheads in a single fabrication operation, allowing for complex multicellular patterns and constructs. The concept of tissue and organ 3D printing, which is being widely explored today, has evolved over years starting with ink-jet bioprinting. Both thermal and piezoelectric DOD printers have been explored for cell bioprinting, but the use of thermal ones has been more prevalent (Cui et al., 2012). In thermal ink-jet printing, while the localized temperature around the heating element can reach between 200–300°C, it lasts for only a few microseconds, and the ejected cells are subjected to a temperature rise of only a couple of degrees above ambient for 2 μ s (Cui et al., 2012; Roth et al., 2004; Cui et al., 2010).

Xu et al. (2005) have highlighted the initial challenges in adapting the piezoelectric mechanism for ink-jet cell printing, which revolve around their higher ink viscosity. Primarily, the frequencies and power employed by commercial piezoelectric printers lie within the same range of vibrating frequencies (15–25 kHz) and power (10–375 W) that are known to disrupt cell membrane and cause cell lysis during sonification (Cui et al., 2012; Xu et al., 2005; Simons et al., 1989; Hopkins, 1991). Adapting piezoelectric printers for less viscous ink to lower the frequency and power would be challenging, since the

resulting ink leakage and mist formation during printing would affect the spatial and feature resolution (Cui et al., 2012; Xu et al., 2005). However, it should be noted that in recent years, other research groups have successfully demonstrated more than 90% viability for piezoelectrically deposited mammalian cells including human osteoblasts, fibroblasts, and bovine chondrocyte cells (Saunders et al., 2005; Saunders et al., 2008; Saunders et al., 2004). Nonetheless, the original investigations into the feasibility of ink-jet bioprinting of live cells were performed on a piezoelectric system. (Wilson and Boland, 2003) used a bioprinter derived from commercially available HP 660C piezoelectric ink-jet printer and custom designed piezoelectric printheads to print viable line patterns (2D) of bovine aortal endothelial cells (BAEC) and smooth muscle cells. In a subsequent study, they printed BAEC aggregates layer-by-layer (3D) in thermosensitive gels with the same printer, and demonstrated that the closely placed cell aggregates could fuse together, which is critical for tissue formation (Boland et al., 2003). Later, they also became the first to use a commercial thermal ink-jet printer (HP 550C and a modified HP 51626a ink cartridge) to create viable patterns of mammalian cells (Chinese Hamster Ovary (CHO) and embryonic motoneuron cells) onto gel substrates (Cui et al., 2010). The viability of printed mammalian cells was found to be in the range of 85–95% for different cell concentrations. The difference in apoptosis ratio and heat shock protein expression level between printed and nonprinted cells was reported to be not statistically significant. Other studies have further investigated the fundamental effects of ink-jet process parameters on viability of different types of cells, and also developed multimaterial composite strategies that combine cells with other biomaterials including proteins, growth factors, and scaffolding polymers (Pepper et al., 2012a; Pepper et al., 2012b; Cui and Boland, 2009; Xu et al., 2006; Chahal et al., 2012). Binder et al. (2011) have provided a primer on DOD ink-jet bioprinting for the research community. Cui et al. (2012) have discussed thermal ink-jet printing from the tissue engineering and regenerative medicine perspective. Derby (2008) has provided a detailed review of ink-jet bioprinting of proteins and hybrid cell-based biomaterials.

3.3.2 PRESSURE-ASSISTED BIOPRINTING

Pressure-assisted bioprinting (PAB) refers to a set of extrusion-based layered manufacturing processes capable of creating digitally controlled 3D patterns and constructs. Biomaterials including polymers and ceramics, proteins and biomolecules, living cells, and growth factors as well as their hybrid structures can be printed using PAB. For printing cells, the bioink is essentially a cell-laden hydrogel of the appropriate viscosity capable of being extruded under pressure through a microscale nozzle orifice or a microneedle at temperatures around 37°C to maintain cell viability. The mechanical integrity of the extruded structures can be controlled through thermal or chemical cross-linking, or multimaterial channel approaches postdeposition. During the process, the biomaterial is contained in a temperature controlled cartridge inside a three axis robotic printhead with a nozzle or microneedle. Deposition takes place by pneumatic pressure, plunger or screw-based extrusion of the material as a continuous filament through the nozzle or microneedle orifice onto a substrate. The substrate can be solid (e.g. culture dish), liquid (e.g. growth media) or a gel based substrate material. The substrate as well as the deposition setup can be contained within a sterile and climate-controlled environment further enabling the use of temperature-sensitive cells and biomaterials. The printhead trajectory is guided by layered data obtained from the digital model of the construct to be laid out. A schematic of the PAB setup is presented in Figure 3.3. The rheological properties of the biomaterial, extrusion temperature, nozzle type used, and applied pressure are the critical parameters that affect the physical and biological characteristics of the printed construct.

**FIGURE 3.3**

Pressure-assisted bioprinting using sodium alginate and calcium chloride as the cross-linking agent to fabricate calcium alginate-based hydrogels. The hydrogels can encapsulate or immobilize any desired cell type within the gel. The precursor solution of sodium alginate and water can be modified to include collagen or other peptides to enhance cellular attachment to promote growth within the gel.

Multiple commercially available multinozzle systems that can be used to deposit biopolymers, cells, and growth factors are currently being marketed by Envisiontec GmbH, Germany (3D Bioplotter) and nScribe, Florida, USA. Other low-cost 3D printing systems, such as those available from Fab@Home, are being modified to fabricate cellular constructs. Traditionally, pressure-assisted deposition processes were being utilized for the fabrication of tissue-engineered scaffolds, since the controlled pore architecture due to the CAD/CAM-controlled layer-by-layer approach was not achievable by traditional scaffold fabrication processes, such as salt leaching and solvent casting. Several studies have focused on the design and optimization of scaffolds using polymers, such as PCL, PLGA, PLLA, PEG, and their blends, and their composites with ceramics, such as hydroxyapatite (HA) and tricalcium phosphate (TCP), have been reported (Vozzi et al., 2002; Wang et al., 2004; Xiong et al., 2001; Landers and Mulhaupt, 2000; Park et al., 2011).

In recent years, with the emergence of the concept of organ printing, the focus has shifted toward direct printing of cell-encapsulated hydrogels. (Yan et al., 2005a; Yan et al., 2005b; Wang et al., 2006; Cheng et al., 2008), and (Xu et al., 2007) used pressure-assisted multisyringe deposition systems to fabricate liver constructs by encapsulating rat hepatocytes in hydrogels including gelatin in conjunction with chitosan, alginate, and fibrinogen. The initial structural support was achieved by thermal cross-linking of gelatin extruded from a low-temperature syringe onto a warmer stage, and the constructs were further strengthened by chemical cross-linking. Favorable cell viability and function results were obtained based on liver tissue markers (urea and albumin production). Although there was difficulty in stabilizing the 3D structure due to enzymatic degradation of the gelatin/chitosan constructs, the process allowed simultaneous deposition of living cells within a biomaterial. Furthermore, (Xu et al., 2009) and (Li et al., 2009) fabricated biomimetic 3D constructs by simultaneous deposition of adipose-derived stem cells and hepatocytes encapsulated in gelatin-based hydrogels. Similarly, Fedorovich et al. have demonstrated the feasibility of multicellular bioprinted constructs incorporating goat multipotent stromal cells (MPSCs), endothelial progenitor cells in Matrigel[®], alginate-based materials for bone grafts (Fedorovich et al., 2001), human mesenchymal stem cells (MSCs), and articular chondrocytes for osteochondral grafts (Fedorovich et al., 2011).

Most recent approaches with pressure-assisted deposition have focused on creating multimaterial multifunctional constructs. For example, (Schuurman et al., 2011) have demonstrated fabrication of hybrid constructs of polycaprolactone (PCL) and chondrocytes-laden alginate. The alginate was cross-linked with calcium chloride postdeposition, but showed relatively good cell viability. Using the same strategy, (Shim et al., 2011) successfully fabricated hybrid constructs containing a PCL/PLGA blend as

the structural polymer alongside collagen containing preosteoblast cells. Using the same polymer and cell-laden hydrogel composite strategy, (Lee et al., 2014a) fabricated a viable auricle using PCL for the structural framework, water-soluble poly-ethylene-glycol (PEG) as a sacrificial material, and chondrocytes/ adipocytes differentiated from adipose-derived stromal cells encapsulated in alginate hydrogel as the biological component. Quantitative analyses showed that the auricular cartilage and earlobe fat can be regenerated while maintaining their inherent functions in different regions of the same structure at the same time by printing chondrocytes and adipocytes separately. (Mannoor et al., 2013) fabricated a bionic ear in the anatomic geometry of a human auricle using silicone as the structural component, bovine chondrocytes-laden alginate as the biological component, and silver nanoparticles infused silicone for electronics. They were able to demonstrate structural integrity and shape retention, >90% viability of the printed chondrocytes, and enhanced auditory sensing for radio frequency reception, all within a single simultaneously printed 3D construct.

3.3.3 LASER-ASSISTED BIOPRINTING

Laser-assisted bioprinting (LAB) is a set of noncontact direct writing processes that utilize a pulsed laser beam to deposit biological materials using cells onto a substrate. Three components are central to most LAB systems—a pulsed laser source, a bioink-coated “ribbon,” and a receiving substrate. Nanosecond lasers with UV or near UV wavelengths are used as the energy source. The “ribbon” is a glass or quartz target plate that is transparent to the laser radiation wavelength and has one side coated with a heat-sensitive bioink consisting of cells either adhered to a biological polymer or uniformly encapsulated within a thin layer of hydrogel. Depending on the optical characteristics of the bioink and the laser wavelength, the system may also contain a laser-absorbing interlayer between the target plate and the bioink to allow viable cell transfer. The receiving substrate positioned below the bioink-coated side of the ribbon is coated with a biopolymer or cell culture medium to maintain cellular adhesion and sustained growth after cell transfer from the ribbon. The pulse of laser causes rapid volatilization at the ribbon’s plate-bioink interface and propels a high-speed jet of the cell-laden bioink onto the receiving substrate.

A schematic of the LAB approach is presented in Figure 3.4. Commonly used LAB processes based on this fundamental working principle include absorbing film-assisted laser-induced forward transfer (AFA-LIFT) or biological laser processing (BioLP) (Hopp et al., 2004; Barron et al., 2005)

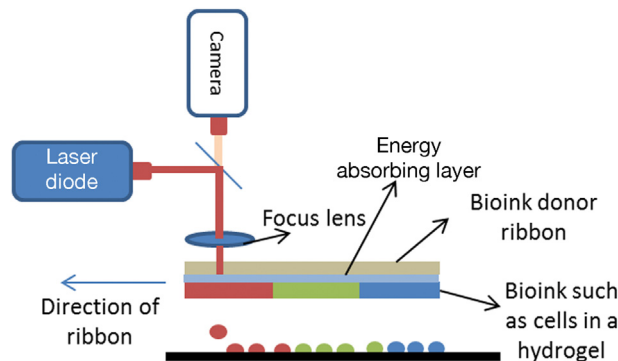


FIGURE 3.4

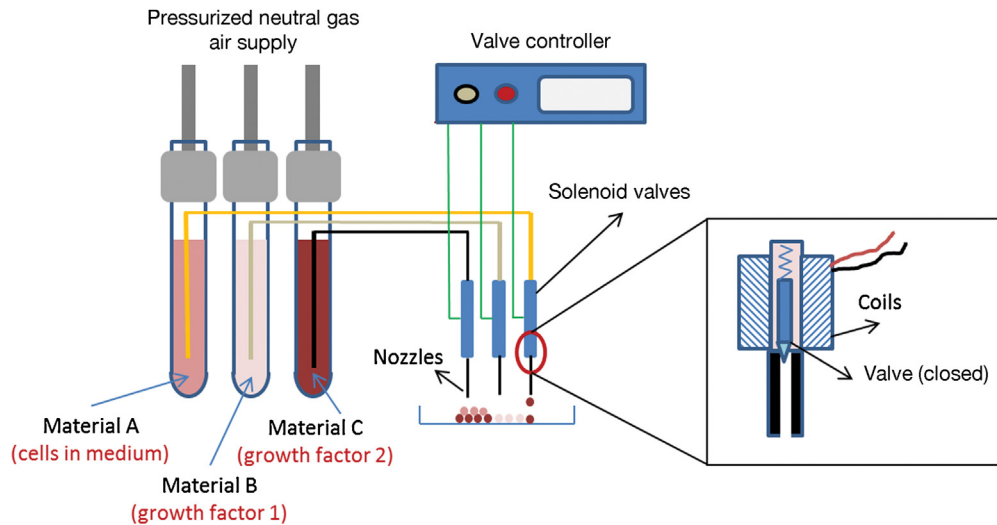
Laser-assisted bioprinting

and matrix-assisted pulsed laser evaporation direct writing (MAPLEDW) (Barron et al., 2004; Patz et al., 2006). The AFA-LIFT and BioLP are bioprinting versions of the laser-induced forward transfer (LIFT) technique which was originally developed for the direct writing of metal features using a high-energy pulsed laser to deposit a metal film on an optically transparent substrate and has also been employed for direct writing of biomolecules (Duocastella et al., 2007). In AFA-LIFT and BioLP, a high-powered pulsed laser is used to vaporize and deposit the bioink onto the substrate. To protect cells from direct exposure to the high-energy laser beam, a sacrificial metal or metal oxide (e.g. Au, Ag, Ti, and TiO₂) thin film (~100 nm) is included at the interface between the target plate and the bioink. The rapid thermal expansion of this interfacial layer due to the high-energy laser pulse propels a small volume of bioink onto the substrate, but with minimal heating of the bioink to prevent cell damage. The BioLP process also utilizes computer-controlled motorized stages and a CCD camera to visualize and focus the laser. Unlike AFA-LIFT and BioLP, the MAPLE-DW process uses a low-power pulsed laser and an interfacial layer of a sacrificial hydrogel such as Matrigel® instead of a metal thin film to accomplish cell transfer. This interfacial layer that acts as an attachment layer for the cells also absorbs the laser energy to prevent it from affecting them. Similar to BioLP, computer-controlled manipulation of the stages coupled with a CCD camera allows for selective cell patterning. Laser-guided direct writing (LGDW) is another commonly used LAB process, but unlike other LAB processes, it does not use a pulsed laser or a print ribbon (Nahmias et al., 2005). Instead, the optical energy of a weakly focused continuous laser is directly used to target and manipulate individual cells from liquid cell suspension onto the substrate. Several parameters related to the laser source, bioink, substrate, interfacial ribbon layer, among others, affect the resolution and performance of LAB processes, and have been described by (Guillemot et al., 2010).

MAPLEDW was one of the first processes used to successfully demonstrate the feasibility of laser-based printing of mammalian cells. Bu et al. (2001) first demonstrated the patterning of Chinese hamster ovary (CHO) cells using the process. Later, Ringeisen et al. (2004) and Barron et al. (2005) printed mouse carcinoma cells (P19), human osteosarcoma (MG-63), and rat cardiac cells with the same process with viability greater than 95%. Recently, (Schiele et al., 2010) have used the MAPLEDW to create viable patterns of human dermal fibroblasts, mouse C2C12 myoblasts, bovine pulmonary artery endothelial (BPAEC), breast cancer (MCF-7), and rat neural stem cells. The AFA-LIFT and BioLP processes have also been successfully used to print various cell types including human osteosarcoma (MG-63), rat Schwann and astroglial and pig lens epithelial cells, BAEC, human umbilical vein endothelial cells (HUVECs), and human umbilical vascular smooth muscle cells (HUVSMC). Invalid source specified. Schiele et al. (2010b) have recently provided a thorough topical review of LAB processes and their applications.

3.3.4 SOLENOID VALVE-BASED PRINTING

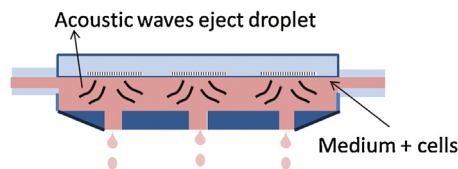
Microdispensing using solenoid valves has seen applications in depositing solder and adhesives onto electronic boards, deposition of optical and electrical polymers, and deposition of biomolecules such as DNA, proteins, and diagnostic reagents. The system has shown itself capable of printing live biological cells for dermal repair, printing mesenchymal stem cells onto tissue well plates, and printing of constructs within a controlled environment. A complete system consists of a fluid reservoir, a solenoid-based dispensing device with droplet volumes ranging from 5 pl to 1 nl droplet quantities, heating elements to control the nozzle head temperature, connections to the pneumatic controller, and an inert gas

**FIGURE 3.5**

Solenoid valve-based bioprinting capable of depositing 20 μ l or higher droplets of living cells and biological molecules.

source. Multiple print head assemblies can be fitted together to improve the throughput of the system. Figure 3.5 shows the schematic of a solenoid-based jetting nozzle. The droplet volume of the printed materials can be controlled by the applied air pressure and the frequency of the solenoid valve open time. Electrical pulse signals sent from the computer can engage or disengage the solenoid leading to droplet ejection from the nozzle. Different nozzle diameters can be attached to the print head to deliver precise quantities of the fluid. The solenoid valve system does not involve heat and is capable of accepting viscous polymers such as collagen and 1–2% sodium alginate. Multiple nozzles can be fitted to the robotic stage to print multiple materials to form a complex heterogeneous construct.

Yoo and coworkers (Lee et al., 2010) reported using this technique for the on-demand fabrication of cellular constructs containing a neural cell line, a fibrin matrix containing a vascular endothelial growth factor (VEGF), and a collagen hydrogel. Since fibrin gel cannot be preloaded into the cartridge, its constituents—fibrinogen, thrombin, and heparin—were separated into two different material cartridges. A third cartridge contained the neural cells to be printed and a fourth cartridge contained a sodium bicarbonate to help in the cross-linking of the collagen gel. With an initial cell density of 1×10^6 cells/ml, each printed droplet of volume 11 ± 0.6 nl contained about 56 ± 9 cells. Reported viability of cells within the 500 μ m thick collagen construct was greater than 93% soon after printing. This work demonstrates the feasibility of precisely placing desired concentrations of VEGF within spatial locations inside the construct to affect cellular behavior, namely proliferation and differentiation, by controlling the time release behavior of these growth factors. In another study using a similar technique, Karande and his team demonstrated the solenoid-based printing technique to engineer human skin. Fibroblasts and keratinocytes representing the epidermis and dermis respectively, along with collagen were bioprinted to showcase the capability of fabricating a complex living system. The printed skin tissue provides applications in topical drug formulation discovery and screening along with designing autologous grafts for wound healing (Lee et al., 2014b).

**FIGURE 3.6**

Acoustic wave-based bioprinting.

3.3.5 ACOUSTIC-JET PRINTING

For applications that require precise placement of single cells, acoustic-based bioprinting is a viable method to deposit picoliter quantities of the medium or hydrogel encapsulating a single cell in a droplet. The ejection of the fluid medium is by focused surface acoustic waves generated by a piezoelectric actuator (such as quartz, lithium tantalite, and lithium niobate) on interdigitated and periodically spaced gold rings. Upon activation by a sinusoidal electrical signal at the same resonant frequency of the device, surface acoustic waves are generated (Figure 3.6). With specially designed printheads, these waves pass through the fluidic environment, in this case, a biomaterial with cells, and are focused to a single point at the fluid-air interface. At the focal point, the waves interfere constructively at the point and the forces exerted by the acoustic radiation will be larger than the surface tension of the fluid leading to the ejection of a droplet from the printhead. The higher the applied frequency, the smaller the droplets generated from a single nozzle. The droplet diameter also depends on the viscosity of the solution contained within the device. For example, a sucrose-dextrose solution can generate droplets ranging from 200 to 3 μm with a respective frequency range of 10 to 100 MHz at a prescribed operational wavelength. The droplet diameter can be customized to the biomolecule being printed. The drop-on-demand feature of the actuation makes for fast deposition speeds of up to 100,000 droplets per second, making it one of the fastest printing methods available. Multiple nozzles and associated acoustic wave generators can be placed periodically to improve throughput rates of fluid ejection. Hence, this method can enable high-throughput studies by patterning a range of biomolecules including RNA, DNA, extracellular matrix proteins, drugs, growth factors, and living cells in microwells (Moon et al., 2010; Gurkan et al., 2014).

3.4 CHALLENGES AND FUTURE DEVELOPMENT OF 3D BIOPRINTING

The eventual goal of all biomedical technologies is to make them clinically available so that patients can benefit from them. All bioprinting processes discussed in this chapter are at different stages of development along that path, but none of them have been translated to FDA-approved clinical applications yet (Table 3.1). By allowing the integration of living and nonliving biomaterials in hybrid structures, bioprinting overcomes several drawbacks associated with previous approaches to tissue fabrication. But despite the significant effort in developing and improving process principles and demonstrating their feasibility for viable cell printing, several supplementary and complementary challenges will need to be addressed to accelerate the successful clinical translation of bioprinting technology, first *in vitro*, and then *in vivo*. As several researchers and experts have noted, thorough characterization of the underlying process mechanisms, their inputs and their outputs will help in the development of machines and systems with low output variability, eventually leading to lower processing costs and improved commercial and clinical potential.

Table 3.1 Comparison between bioprinting processes

Process	Common subprocesses/ mechanisms	Mammalian cell/tissue types	Typical hydrogels/ bioinks/biopaper	Suitability for applications			Salient features		
				Therapeutic	Drug-testing and disease modeling	Cell interaction studies	Spatial resolution	Single cell control	Throughput
Ink-jet Bioprinting	• Thermal • Piezoelectric	• Ovary cells (Delaney et al., 2009; Cui et al., 2012) • Motoneurons (Cui et al., 2012) • Endothelial cells (Saunders et al., 2005) • Osteoblasts (Xu et al., 2005; Hopkins, 1991) • Fibroblasts (Simons et al., 1989; Hopkins, 1991) • Chondrocytes (Xu et al., 2005; Hopkins, 1991) • Smooth muscle cells	• DPBS (Delaney et al., 2009; Cui et al., 2012) • Collagen (Delaney et al., 2009; Cui et al., 2012; Saunders et al., 2005) • Soy agar (Cui et al., 2012) • Culture Media (Xu et al., 2005; Simons et al., 1989; Saunders et al., 2005) • Matrigel (Saunders et al., 2005) • Alginate	L	H	M	M	L	H

(Continued)

Table 3.1 Comparison between bioprinting processes (*cont.*)

Process	Common subprocesses/ mechanisms	Mammalian cell/tissue types	Typical hydrogels/ bioinks/biopaper	Suitability for applications			Salient features		
				Therapeutic	Drug-testing and disease modeling	Cell interaction studies	Spatial resolution	Single cell control	Throughput
Pressure-assisted Bioprinting	- Air-pressure - Screw-extrusion	- Hepatocytes (Xiong et al., 2001; Landers and Mulhaupt, 2000; Park et al., 2011; Yan et al., 2005a; Yan et al., 2005b; Cheng et al., 2008; Fedorovich et al., 2001) - Adipose-derived stromal cells (Wang et al., 2006; Cheng et al., 2008) - Multipotent stromal cells (Xu et al., 2007; Xu et al., 2009) - Endothelial progenitor cells (Xu et al., 2007) - Chondrocytes (Xu et al., 2009; Li et al., 2009; Schuurman et al., 2011) - Pre-osteoblasts (Fedorovich et al., 2001) - Adipocytes (Fedorovich et al., 2001) - Ovary cells - Smooth muscle cells - Fibroblasts	- Gelatin (Xiong et al., 2001; Landers and Mulhaupt, 2000; Park et al., 2011; Yan et al., 2005a; Yan et al., 2005b; Wang et al., 2006; Cheng et al., 2008; Fedorovich et al., 2001) - Alginate (Xiong et al., 2001; Landers and Mulhaupt, 2000; Park et al., 2011; Xu et al., 2007; Xu et al., 2009; Li et al., 2009; Fedorovich et al., 2011; Schuurman et al., 2011) - Chitosan (Landers and Mulhaupt, 2000; Yan et al., 2005a; Cheng et al., 2008) - Fibrinogen (Yan et al., 2005b; Wang et al., 2006; Cheng et al., 2008) - Matrigel Xu et al., 2007 - Collagen (Fedorovich et al., 2001) - Hyaluronic acid (Fedorovich et al., 2001) - Agarose	H	H	L	L	L	H

Laser-assisted Bioprinting	• MAPLE-DW • AFA-LIFT/BioLP • LG-DW	• Epithelial cells (Lee et al., 2014a) • Osteosarcoma (Mannoor et al., 2013; Hopp et al., 2004; Barron et al., 2005) • Cardiac cells (Barron et al., 2005) • Neurons (Barron et al., 2004) • Spinal cord cells (Ringeisen et al., 2004) • Endothelial cells (Schiele et al., 2010a; Moon et al., 2010) • Hepatocytes (Schiele et al., 2010a) • Ovary cells (Lee et al., 2010) • Carcinoma (Lee et al., 2014b) • Fibroblasts (Moon et al., 2010; Gurkan et al., 2014) • Myoblasts (Moon et al., 2010) • Neural stem cells (Moon et al., 2010) • Smooth muscle cells	• Gelatin (Lee et al., 2014a; Schiele et al., 2010a; Gurkan et al., 2014) • Matrigel (Mannoor et al., 2013; Hopp et al., 2004; Barron et al., 2005; Barron et al., 2004; Schiele et al., 2010a; Lee et al., 2010; Lee et al., 2014b; Moon et al., 2010)	L	H	H	M	M	M
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(Continued)

Table 3.1 Comparison between bioprinting processes (cont.)

Process	Common subprocesses/mechanisms	Mammalian cell/tissue types	Typical hydrogels/bioinks/biopaper	Suitability for applications			Salient features		
				Therapeutic	Drug-testing and disease modeling	Cell interaction studies	Spatial resolution	Single cell control	Throughput
Solenoid-based Printing	N/A	• Neural cells (Lee et al., 2010) • Fibroblasts (Lee et al., 2014b) • Keratinocytes (Lee et al., 2014b)	• Fibrin (Lee et al., 2010) • Collagen (Lee et al., 2010; Lee et al., 2014b)	H	H	L	M	M	M
Acoustic-jet Printing	N/A	• Embryonic stem cells (Moon et al., 2010) • Fibroblasts (Moon et al., 2010) • Cardiomyocytes (Moon et al., 2010) • Raji cells (Moon et al., 2010) • Breast cancer cells (Gurkan et al., 2014) • Embryonic kidney cells [B]	• Agarose (Moon et al., 2010) • PBS (Moon et al., 2010) • Dextran (Gurkan et al., 2014)	L	M	H	M	H	H

L: Low; M: Medium; H: High.

We have highlighted here several issues that must be addressed by the 3D bioprinting research community for the wide-scale adoption of this base technology for several therapeutic and nontherapeutic applications.

Lack of digital models: Data drive the bioprinting machines. Currently there is a lack of software architecture with necessary design tools to engineer tissue and organ systems. New data structures must be developed to capture the heterogeneous information necessary to define such living products. Virtually defining the placement of cells, biomaterials, and biological molecules will lead to designs that are robust. The digital definition can also translate to drive all downstream manufacturing operations. Conventional design software revolutionized methods through which automobiles, airplanes, and consumer and medical products are designed and manufactured. Similar software design platforms must be available if the benefits of bioprinting are to be widely adopted by the research and industrial communities. On a similar note, designers should also be presented with tools to simulate engineered tissue/organoids to pursue what-if analysis scenarios. Tissue systems constantly remodel and change over time. Tools to help predict end function, stability, and efficacy will be necessary for the wide-spread adoption of bioprinting technologies.

Biomaterials are limited, proprietary, and expensive: Another key limitation is the limited class of biomaterials in which the cells are encapsulated. While cell-free scaffolds are printed in a variety of biomaterials, there has not been much development into designing newer biomaterials able to accommodate the encapsulated cells and the printing process in general. While it is generally understood the cells will secrete their own extracellular matrix over time, the initial biomaterial plays an important role in producing the right microenvironment for cells to be accommodated in their new engineered environment. Polymers such as calcium alginate, poly-lactic-glycolic acid (PLGA), and poly-ethylene glycol-diacrylate (PEGDA) are some of the most common polymers used in the 3D bioprinting process. Most polymers used for bioprinting are synthesized in laboratories or are available in commercial quantities that can be cost-prohibitive. There is a need to expand the library of biomaterials to be used for the several bioprinting machines available. Similar to established metals and polymers with their defined properties, new classes of printable biomaterials must be developed to produce engineered tissue and organs.

Lack of adequate cell loading and uniform cell distribution: Engineered constructs of the heart or liver will require millions of cells packed in given volume of a printed construct if they are to function in a physiological manner. Current bioprinting techniques are limited to less than 10 M cells/ml. Improving cell density to reach greater than 50 to 100 M cells/ml will be necessary for proper tissue and organ function. Long fabrication times can lead to settling down of the cells in the printhead chamber. This will lead to nonuniform distribution of cells resulting in inconsistent results. For bioprinting technologies that specify one or two cells per droplet, improving the reliability of cells contained within a droplet is necessary for improving the robustness of the process.

Material development and standardization: Bio-inks are an integral part of the bioprinting technology. It is not the bioprinting process parameters alone, but the material–process interactions that govern the viability and success of the resultant constructs. Hence, developing appropriate bioinks and comprehensively characterizing their rheological, mechanical, and biological characteristics is critical to the success of bioprinting. It is accepted that this development and characterization will have to be cell/tissue- and process-specific. Standardization of the living as well as nonliving bioink components and their sources is also of equal importance, not only from the final production perspective, but also during the process development stage, since any variability in the bioink characteristics ultimately affects the variability of

the bioprinting process output. For example, lot-to-lot variability in the growth media can affect the viability of living and biomolecular components of the bioink post printing. Similarly, the viability of fabricated structures can be affected by the source of cells and their storage method prior to printing, even when the bioprinting process parameters are held constant. During the development and characterization of a bioprinting process for allogenic applications, it is critical that the appropriateness of the selected cell source be determined from an application perspective, and its characteristics be thoroughly analyzed and documented. Concepts such as crowd sourcing can be used during the research and development phase to eliminate variability caused by the bioink material.

Measurement and in-process monitoring technologies: A key technology gap to advance bioprinting technologies for engineered tissue is the ability to monitor in real time the process of printing cells and biological moieties. While optical methods are being utilized to monitor droplets and printed constructs, currently there are limited options available to nondestructively evaluate the “living” components within the printed constructs. For example, in the scale-up production of printed cellular constructs, how do we monitor both the placement and functionality of cells within the biomaterial? The problem is even more challenging when encapsulated in 3D hydrogel. Real-time label-free monitoring methods must be developed to advance the scalability and integration of the bioprinting process to economically viable production. The data generated can also help to identify critical process parameters and help implement statistical process control for the bioprinting processes. Ultimately these technologies will be needed to help scale-up or scale-out the process to help meet the demand for customized tissue- and organs-on-demand.

3.5 Conclusion

Undoubtedly, the ability to accurately place cells and cellular constructs to form engineered tissue and organoid systems by the definition of a digital model is powerful. The technologies presented offer viable and high-throughput approaches to printing cells and hydrogels in a biocompatible and cell-friendly environment. Spatial control, precise placement of multiple cell types, and high-throughput speed when compared to manual methods are clearly the biggest advantages of the 3D bioprinting technology. When compared to microfabrication techniques such as microstamping and micromolding, the biggest advantage of 3D bioprinting is the ability to define complex interior architecture in true three-dimensions due to the layer by layer additive manufacturing approach taken by most bioprinting technologies. This bottom-up technology bridges microscale and mesoscale definition of engineered tissue, thereby offering the possibility of addressing the challenge of building thick tissues and organ systems.

An immediate application of 3D bioprinting are the use of these systems to create disease models to study pathophysiology, as complex *in vitro* tissue models to screen for new therapeutic drugs and as systems to help achieve engineered meat and leather products. Maturation of these applications of bioprinting will see some of the groundwork being laid to address the challenges highlighted in the previous section. Lab-grown organs and functional tissue intended for human implantation will likely see another decade of fundamental research activity before they have the possibility to become real application scenarios. The future success and commercial viability of the process will depend on efforts to address the challenges to adoption of bioprinting. The systems have the potential to be widely adopted and integrated within conventional cell culture laboratories. They provide a new avenue for researchers to ask research questions in the third dimension, thereby replicating the dynamics of *in vivo* cellular physiology and microenvironment. Bioprinting also has

clear applications in regenerative medicine with the ability of these machines to be used in clinical room settings for achieving personalized tissue-on-demand and replacement organs. There are challenges to achieving this vision, but as advancements are made in hardware and software technology coupled with an increased understanding of cellular behavior in the engineered environment, bioprinting will be a viable new biomanufacturing technology.

References

- Barron, J. A., Ringeisen, B. R., Kim, H., Spargo, B. J., & Chrisey, D. B. (2004). Application of laser printing to mammalian cells. *Thin Solid Films*, 1, 383–387.
- Barron, J., Wu, P., Adouceur, H., & Ringeisen, B. (2005). Biological laser printing: a novel technique for creating heterogeneous 3-dimensional cell patterns. *Ann Biomed Eng*, 6(2), 121–130.
- Binder, K. W., Allen, A. J., Yoo, J. J., & Atala, A. (2011). Drop-on-demand inkjet bioprinting: a primer. *Gene Therapy and Regulation*, 6(1), 33–49.
- Boland, T., Mironov, V., Gutowska, A., Roth, E. A., & Markwald, R. R. (2003). Cell and organ printing 2: fusion of cell aggregates in three-dimensional gels. *Anatomical record Part A, Discoveries in Molecular, Cellular, and Evolutionary Biology*, 272, 497–502.
- Bu, R., Wu, P. C. J., Brooks, M., Bubbs, D., Wu, H., Piqué, A., B, S., R, M., & Chrisey, D. B. (2001). The deposition, structure, pattern deposition, and activity of biomaterial thin-films by matrix-assisted pulsed-laser evaporation (MAPLE) and MAPLE direct write. *Thin Solid Films*, 607–614.
- Chahal, D., Ahmadi, A., & Cheung, K. C. (2012). Improving piezoelectric cell printing accuracy and reliability through neutral buoyancy of suspensions. *Biotechnology and Bioengineering*, 109, 2932–2940.
- Cheng, J., Lin, F., Liu, H., Yan, Y., Wang, X., Zhang, R., & Xiong, Z. (2008). Rheological properties of cell-hydrogel composites extruding through small-diameter tips”. *Journal of Manufacturing Science and Engineering*, 130, 021014.
- Cui, X., & Boland, T. (2009). Human microvasculature fabrication using thermal ink-jet printing technology. *Biomaterials*, 30, 6221–6227.
- Cui, X., Dean, D., Ruggeri, Z. M., & Boland, T. (2010). Cell damage evaluation of thermal ink-jet printed Chinese hamster ovary cells. *Biotechnology and Bioengineering*, 106, 963–969.
- Cui, X., Boland, T., D’Lima, D. D., & Lotz, M. K. (2012). Thermal inkjet printing in tissue engineering and regenerative medicine. *Recent Patents on Drug Delivery & Formulation*, 6(2), 149–155.
- Delaney, J. T., Jr., Smith, P. J., & Schubert, U. S. (2009). Inkjet printing of proteins. *Soft Matter*, 5, 4866–4877.
- Derby, B. (2008). Bioprinting: ink-jet printing proteins and hybrid cell-containing materials and structures. *Journal of Materials Chemistry*, 18, 5717–5721.
- Derby, B. (2012). Printing and prototyping of tissues and scaffolds. *Science*, 338, 921.
- Duocastella, M., Colina, M., Fernandez-Pradas, J. M., Serra, P., & J.L, M. (2007). Study of the laser-induced forward transfer of liquids for laser bioprinting. *Applied Surface Science*, 253, 7855–7859.
- Fedorovich, N. E., Wijnberg, H. M., Dhert, W. J., & Alblas, J. (2011). Distinct tissue formation by heterogeneous printing of osteo- and endothelial progenitor cells. *Tissue Engineering Part A*, 17, 2113–2121.
- Fedorovich, N. E., Schuurman, W., Wijnberg, H. M., Prins, H. J., Van Weeren, P. R., & Malda, J. (2011). J. Alblas and W. J. Dhert, Biofabrication of osteochondral tissue equivalents by printing topologically defined, cell-laden hydrogel scaffolds. *Tissue Engineering Part C: Methods*, 18(1), 33–44.
- Guillemot, F., Souquet, A., S, C., & B, G. (2010). Laser-assisted cell printing: principle, physical parameters versus cell fate and perspectives in tissue engineering. *Nanomedicine*, 5(3), 507–515.
- Gurkan, U. A., Assal, R. E., Yildiz, S. E., Sung, Y., Alexander, W. P. K., Trachtenberg, J., & Demirci, U. (2014). Engineering anisotropic biomimetic fibrocartilage microenvironment by bioprinting mesenchymal stem cells in nanoliter gel droplets. *Mol. Pharmaceutics*.

- Hopkins, T. R. (1991). In R. Seetharam, & S. K. Sharma (Eds.), *Purification and analysis of recombinant proteins* (p. 69). New York: Marcel Dekker.
- Hopp, B., Smausz, T., Antal, Z., Kresz, N., Bor, Z., & D.B, C. (2004). Absorbing film assisted laser induced forward transfer of fungi (*Trichoderma conidia*). *Journal of Applied Physics*, 96, 3478–3481.
- Landers, R., & Mulhaupt, R. (2000). Desktop manufacturing of complex objects, prototypes, and biomedical scaffolds by means of computer-assisted design combined with computer-guided 3D plotting of polymers and reactive oligomers. *Macromolecular Materials and Engineering*, 282, 17–21.
- Lee, Y.-B., Polio, S., Lee, W., Dai, G., Menon, L., Carrol, R. S., & Yoo, S.-S. (2010). Bioprinting of collagen and VEGF-releasing fibrin gel scaffolds for neural stem. *Experimental Neurology*, 223, 645–652.
- Lee, J.-S., Hong, J. M., Jung, J. W., Shim, J.-H., Oh, J.-H., & Cho, D.-W. (2014a). 3D printing of composite tissue with complex shape applied to ear regeneration. *Biofabrication*, 6, 024103.
- Lee, V., Singh, G., Trasatti, J., Bjornsson, C., Xu, X., Tran, T. N., Yoo, S.-S., Dai, G., & Karande, P. (2014b). Design and fabrication of human skin by 3D bioprinting. *Tissue Engineering Part C: Methods*, 20(6), 473–484.
- Li, S., Xiong, Z., Wang, X., Yan, Y., Liu, H., & Zhang, R. (2009). Direct fabrication of a hybrid cell/hydrogel construct by a double nozzle assembling technology. *Journal of Bioactive and Compatible Polymers*, 24, 249–265.
- Lipson, H. (2013). *Fabricated: The New World of 3D Printing*. Wiley.
- Mannoor, M. S., Jiang, Z., James, T., Kong, Y. L., Malatesta, K. A., Soboyejo, W. O., Verma, N., Gracias, D. H., & McAlpine, M. C. (2013). 3D printed bionic ears. *Nano Letters*, 13, 2634–2639.
- Moon, S., Hasan, S. K., Song, Y. S., Khademhosseini, A., & Demirci, U. (2010). Layer by layer three-dimensional tissue. *Tissue Engineering: Part C*, 16(1), 157–165.
- Nahmias, Y., Schwartz, R. E., Verfaillie, C. M., & D.J, O. (2005). Laser-guided direct writing for three-dimensional tissue engineering. *Biotechnology and Bioengineering*, 92, 129–136.
- Okamoto, T., Suzuki, T., & Yamamoto, N. (2000). Microarray fabrication with covalent attachment of DNA. *Nature Biotechnology*, 18, 438–441.
- Park, S. A., Lee, S. H., & Kim, W. D. (2011). Fabrication of porous polycaprolactone/hydroxyapatite (PCL/HA) blend scaffolds using a 3D plotting system for bone tissue engineering. *Bioprocess and Biosystems Engineering*, 34(4), 505–513.
- Patz, T. M., Doraiswamy, A., Narayan, R. J., He, W., Zhong, Y., Bellamkonda, R., Modi, R., & Chrisey, D. B. (2006). Three-dimensional direct writing of B35 neuronal cells. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, 78(1), 124–130.
- Pepper, M. E., Seshadri, V., Burg, T. C., Burg, K. J., & Groff, R. E. (2012a). Characterizing the effects of cell settling on bioprinter output. *Biofabrication*, 4, 011001.
- Pepper, M. E., Groff, R. E., Cass, C. A., Mattimore, J. P., Burg, T., & Burg, K. J. (2012b). A quantitative metric for pattern fidelity of bioprinted cocultures. *Artificial Organs*, 36, E151–162.
- Reis, N., Ainsley, C., & Derby, B. (2005). Ink-jet delivery of particle suspensions by piezoelectric droplet ejectors. *Journal of Applied Physics*, 97(9), 094903.
- Ringeisen, K. H., Barron, B. R. J., Krizman, D., Chrisey, D., & Jackman S, A. R. S. B. (2004). Laser printing of pluripotent embryonal carcinoma cells. *Tissue Eng*, 10(3–4), 483–491.
- Roth, E. A., Xu, T., Das, M., Gregory, C., Hickman, J. J., & Boland, T. (2004). Inkjet printing for high-throughput cell patterning. *Biomaterials*, 25, 3707–3715.
- Saunders, R. E., Bosworth, L., Gough, J. E., Derby, B., & Reis, N. (2004). Selective cell delivery for 3D tissue culture and engineering. *European Cells and Materials*, 7(S1), 84–85.
- Saunders, R., Gough, J., & Derby, B. (2005). *Inkjet printing of mammalian primary cells for tissue engineering applications*. Boston: Nanoscale materials science in biology and medicine.
- Saunders, R. E., Gough, J. E., & Derby, B. (2008). Delivery of human fibroblast cells by piezoelectric drop-on-demand ink-jet printing. *Biomaterials*, 29(2), 193–203.
- Schiele, N. R., Chrisey, D. B., & D.T, T. (2010a). Gelatin-based laser direct-write technique for the precise spatial patterning of cells. *Tissue Engineering Part C: Methods*, 17(3), 289–298.

- Schiele, N. R., Corr, D. T., Huang, Y., Raof, N. A., Y, X., & Chrisey, D. B. (2010b). Laser-based direct-write techniques for cell printing. *Biofabrication*, 2(3).
- Schuurman, W., Khristov, V., Pot, M. W., Van Weeren, P. R., Dhert, W. J., & Malda, J. (2011). Bioprinting of hybrid tissue constructs with tailorable mechanical properties. *Biofabrication*, 3, 021001.
- Seerden, K. A. M., Reis, N., Evans, J. R. G., Grant, P. S., Halloran, J. W., & Derby, B. (2001). Ink-jet printing of wax-based alumina suspensions. *Journal of the American Ceramic Society*, 84(11), 2514–2520.
- Shim, J.-H., Kim, J. Y., & Park, M. (2011). J. Park and D.-W. Cho, Development of a hybrid scaffold with synthetic biomaterials and hydrogel using solid freeform fabrication technology. *Biofabrication*, 3, 034102.
- Simons, K. R., Morton, R. J., Mosier, D. A., Fulton, R. W., & Confer, A. W. (1989). Comparison of the *Pasteurella haemolytica* A1 envelope proteins obtained by two cell disruption methods. *Journal of Clinical Microbiology*, 27(4), 664–667.
- Valerie Liu Tsang, S. N. B. (2004). Three-dimensional tissue fabrication. *Advanced Drug Delivery Reviews*, 56, 1635–1647.
- Vozzi, G., Previti, A., De Rossi, D., & Ahluwalia, A. (2002). Microsyringe-based deposition of two-dimensional and three-dimensional polymer scaffolds with a well-defined geometry for application to tissue engineering. *Tissue Engineering*, 8(6), 1089–1098.
- Wang, F., Shor, L., Darling, A., Khalil, S., Sun, W., Güçeri, S., & Lau, A. (2004). Precision extruding deposition and characterization of cellular poly- ϵ -caprolactone tissue scaffolds. *Rapid Prototyping Journal*, 10(1), 42–49.
- Wang, X., Yan, Y., Pan, Y., Xiong, Z., Liu, H., Cheng, J., Liu, F., Wu, R., Zhang, R., & Lu, Q. (2006). Generation of three-dimensional hepatocyte/gelatin structures with rapid prototyping system. *Tissue Engineering Part A*, 12, 83–90.
- Wilson, W. C., Jr., & Boland, T. (2003). Cell and organ printing 1: protein and cell printers. *The Anatomical Record Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology*, 272A(2), 491–496.
- Xiong, Z., Yan, Y., Zhang, R., & Sun, L. (2001). Fabrication of porous poly(l-lactic acid) scaffolds for bone tissue engineering via precise extrusion. *Scripta Materialia*, 45(7), 773–779.
- Xu, T., Jin, J., Gregory, C., Hickman, J. J., & Boland, T. (2005). Inkjet printing of viable mammalian cells. *Biomaterials*, 26(1), 93–99.
- Xu, T., Gregory, C. A., Molnar, P., Cui, X., Jalota, S., Bhaduri, S. B., & Boland, T. (2006). Viability and electrophysiology of neural cell structures generated by the ink-jet printing method. *Biomaterials*, 27, 3580–3588.
- Xu, W., Wang, X., Yan, Y., Zheng, W., Xiong, Z., Lin, F., Wu, R., & Zhang, R. (2007). Rapid prototyping three-dimensional cell/gelatin/fibrinogen constructs for medical regeneration. *Journal of Bioactive and Compatible Polymers*, 22, 363–377.
- Xu, M., Yan, Y., Liu, H., Yao, R., & Wang, X. (2009). Controlled adipose-derived stromal cells differentiation into adipose and endothelial cells in a 3D structure established by cell-assembly technique. *Journal of Bioactive and Compatible Polymers*, 24, 31–47.
- Yan, Y., Wang, X., Xiong, Z., Liu, H., Liu, F., Lin, F., Wu, R., Zhang, R., & Lu, Q. (2005a). Direct construction of a three-dimensional structure with cells and hydrogel. *Journal of Bioactive and Compatible Polymers*, 20, 259–269.
- Yan, Y., Wang, X., Pan, Y., Liu, H., Cheng, J., Xiong, Z., Ln, F., Wu, R., Zhang, R., & Lu, Q. (2005b). Fabrication of viable tissue-engineered constructs with 3D cell-assembly technique. *Biomaterials*, 26(29), 5864–5871.

The Power of CAD/CAM Laser Bioprinting at the Single-Cell Level: Evolution of Printing

Jayant Saksena¹, S.C. Sklare¹, Theresa B. Phamduy², Yong Huang³ and Douglas B. Chrisey¹

¹*Department of Physics and Engineering Physics, Tulane University, New Orleans, LA, United States*

²*Department of Biomedical Engineering, Tulane University, New Orleans, LA, United States*

³*Department of Mechanical & Aerospace Engineering, University of Florida, Gainesville, FL, United States*

4.1 INTRODUCTION

Single-cell deposition methodologies onto homogeneous two-dimensional (2D) and into three-dimensional (3D) environments with high accuracy and reproducibility are currently entering a new stage of maturity aided by advances in systems engineering, based on a critical mass of technological developments. Coupling computer-aided design (CAD) and computer-aided manufacturing (CAM) principles with the advantages of laser systems enables researchers to create reproducible or iteratively varying biological constructs with single-cell precision. This chapter illustrates the importance of single-cell deposition and introduces laser-assisted bioprinting as a viable method of printing individual cells. Specifically, the following topics will be discussed: advantages of laser-assisted bioprinting systems, examples of laser systems adapted for cell printing, the technology and basic physics behind these systems, mechanistic modeling of laser-assisted cell transfer and several case studies illustrating the importance of printing on an individual cell basis. In particular, we showcase matrix-assisted pulsed-laser evaporation direct write (MAPLE-DW) as the premiere laser-assisted, nozzle-free, and contactless printing method. We focus on recent enhancements in MAPLE-DW: potential scalability, single-cell deposition, ease-of-use, and control of environmental parameters (such as temperature and humidity).

4.1.1 DIRECT CONTACT VERSUS DIRECT WRITE FOR SINGLE-CELL PRINTING

Laser-assisted bioprinting possesses inherent advantages for single-cell applications, but it is not the only method to demonstrate single-cell resolution deposition. Four of the principal categories of bioprinting methods, (1) extrusion bioprinting, (2) inkjet bioprinting, (3) nanocontact bioprinting, and (4) laser-assisted bioprinting, can be further classified as direct-contact (DC) or direct-write (DW) mechanisms. DC methods, such as microcontact patterning, load cells suspended in media onto a “stamp” and then bring that “inked” surface in contact with the desired substrate surface. This prints an entire pattern at once, as opposed to being built over time. Stamps are *a priori* patterns that require new molds for new patterns. After the suspension is applied to the stamp, there is no way to select certain cells or

groups of cells for printing. Thus, the nature of cell distribution in suspension determines the probabilistic cell number per print area. DW methods, such as inkjet printing and laser-assisted transfer, use “bottom-up techniques” that generate whole constructs on a subunit-by-subunit basis. DW methods can be combined with CAD/CAM techniques to facilitate the controlled transfer of biological “voxels” (volumetric pixels) containing the desired cells. By designing blueprints and depositing biological voxels accordingly, researchers can generate 2D arrays or layer 2D patterns to create 3D constructs. However, laser-assisted cell transfer is distinct from other DW methods for single-cell printing applications.

Unambiguous scientific conclusions rely on the reproducibility of experiments to test hypotheses and prove statistical significance. Single-cell printing reduces sample-to-sample variability and permits clear quantification in biological studies by repeating addressable units (voxels) to create cell or tissue constructs. Inkjet and extrusion methods that rely on nozzle-ejected droplets and blind laser-assisted methods that depend on laser-material interactions for droplet formation are capable of printing one cell at a time on average. This average and reproducibility depend on the probabilistic localization of only a single cell within the ejection volume (Barron, Krizman, & Ringeisen, 2005; Liberski, Delaney, & Schubert, 2011). Caution should be taken, however, as voxel-to-voxel reproducibility necessarily varies due to inherent cell-to-cell differences. In addition, the ability to select cells during DW procedures produces less variance compared to blind DW methods simply by enabling the end-user to visibly target cells. Moreover, the impact of scientific conclusions increases inversely with the number of cells written with single-cell deposition being paramount.

Rather than rely on distribution statistics, MAPLE-DW and other select DW techniques provide, *in situ*, real-time optical monitoring and recording to enable users to select cells for printing and confirm deposition onto the substrate. This distinguishing feature provides feedback to determine precision in cell placement, relative to the target location. MAPLE-DW spatial resolution is $\pm 5 \mu\text{m}$ (Schiele, Chrisey, & Corr, 2011). By using a camera-equipped, laser-assisted DW system, one is able to reduce voxel-to-voxel variation, monitor deposition in real-time, and achieve tight control in spatial printing. This platform for cell printing enables researchers to explore numerous biological systems, including stem cell niches, cancer invasion, and neuron manipulation/functionalization using functional testing platforms, such as isolated-node single-cell arrays, network-level single-cell arrays, integrated single cells, and 3D cellular invasion models.

4.2 BASICS OF LASER-ASSISTED PRINTING: OVERVIEW OF SYSTEMS AND CRITICAL ANCILLARY MATERIALS

4.2.1 LASER-ASSISTED CELL TRANSFER SYSTEM COMPONENTS

Variations and generational iterations of laser-based cell transfer bioprinting systems are based on similar principles to those of laser-induced forward transfer (LIFT) for inorganic electronic materials (Piqué, Chrisey, & Auyeung, 1999). The four most common laser bioprinting systems are LIFT, absorbing film-assisted LIFT (AFA-LIFT), biological laser processing (BioLP), and MAPLE-DW. They all utilize optically transparent “ribbon” disks, biopolymer-coated receiving substrates, beam delivery optics, imaging and pulsed-laser systems, but contain variations in ribbon processing and laser characteristics for optimal laser-material interaction. Auxiliary components include *in situ* imaging devices, laser beam energy meters and up to three computer-controlled stages. A complete

survey of laser-assisted printing systems is provided by [Phamduy, Corr, and Chrisey \(2010\)](#). Two specific system schemes, AFA-LIFT and MAPLE-DW, are highlighted here.

The laser-transparent disks, termed “print ribbons,” are analogous to ink ribbons historically utilized in typewriters. By analogy, ribbons are loaded on one side with ink, as the optically transparent print ribbon disks are coated on one side with bioink, which contains cells. Cells either adhere to a biopolymer coating on the disk ribbon or are suspended in a sacrificial matrix layer, such as cell culture media or low viscosity biopolymer (typically a hydrogel). In AFA-LIFT, there may be an additional metallic layer between the ribbon surface and cell-embedded matrix. This sacrificial layer acts as an absorption medium intended to reduce the adverse effects of laser radiation on cells and facilitate the conversion of light energy to mechanical energy. In systems with UV or near-UV lasers, this is a laser-absorbing biopolymer layer. However, in high-power (longer wavelengths with longer pulse widths) laser systems, this layer is often made of gold or titanium. Potential cytotoxic effects exist from the volatilization of the sacrificial metal layer, which leads to potential nanoparticulate inclusion during droplet ejection ([Lewinski, Colvin, & Drezek, 2008](#); [Smausz et al., 2006](#)). In addition, the opaque metal layer blocks visualization of cells on the ribbon.

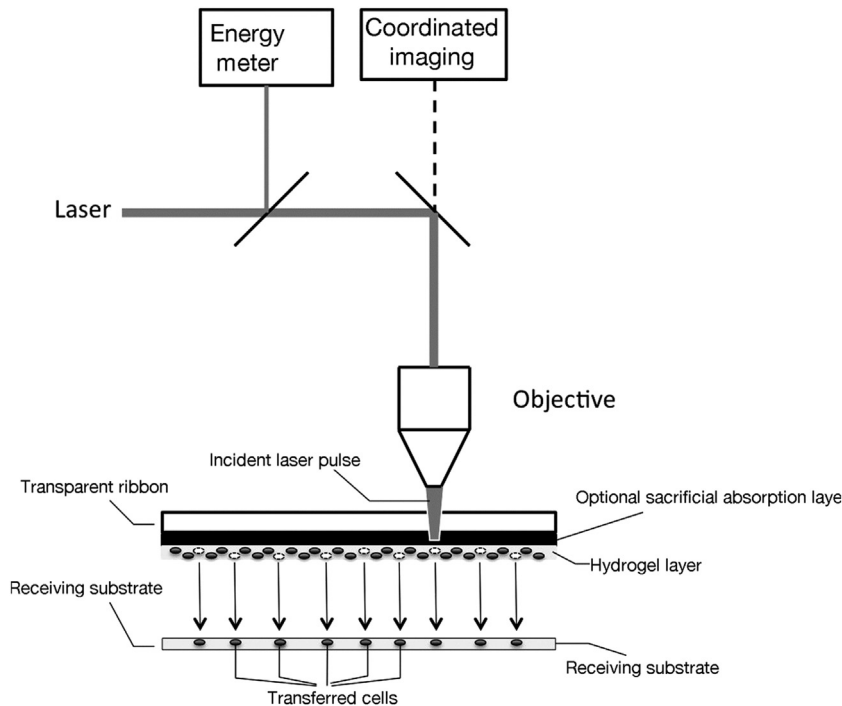
MAPLE-DW positions the print ribbon on planar motorized stages in conjunction with a coordinated imaging system, which allows researchers to traverse the ribbon and select individual cells for printing in real-time. For single-cell transfer applications, print ribbons must have enough inter-cellular spatial separation so that only one cell is situated within the transfer area. The ability to visually target individual cells by way of coordinated imaging and a low cell density on print ribbon removes the element of volumetric probability, reducing droplet-to-droplet variation.

In MAPLE-DW, the substrate is mounted on a tri-axial motorized stage positioned below the ribbon, separated by a small gap of approximately 700–2000 μm wide ([Patz et al., 2006](#)). There is a critically important coating on the receiving face of the substrate composed of a hydrogel enriched with nutrients, for example, a gelatin-based mixture containing cell culture medium. The substrate coatings are carefully chosen to cushion cell impact, maintain a moist environment for cells during printing at ambient conditions, and promote cell adhesion when appropriate. Additional requirements of substrate coatings are discussed later in this section.

Single laser pulses are focused at the print ribbon-cell suspension medium (or intermediate absorption layer) interface to achieve noncontact transfer. Each laser pulse causes localized, rapid evaporation of the support medium and the formation of a vapor bubble. The expanding bubble forces a volume of material to be ejected from the cellular suspension layer. The mechanism of bubble formation and material ejection are detailed later in this chapter and a schematic representation is given in [Fig. 4.1](#). In general, systems that use metallic absorption layers (e.g., LIFT, AFA-LIFT, BioLP) also utilize high-power lasers (typically KrF). Low power, high-energy excimer UV lasers (typically ArF) are used in MAPLE-DW. In general, higher laser pulse energies lead to increased impact force during cell deposition onto the receiving substrate ([Patz et al., 2006](#)). When coupled with a viscous receiving medium, this method can be used to create 3D constructs with adherent cells initially deposited at variable depths. As cells may be injured due to impact and DNA damage from laser irradiation, the variation in laser energy must be minimized.

4.2.2 ABSORBING FILM-ASSISTED LASER-INDUCED FORWARD TRANSFER

AFA-LIFT is a modified version of LIFT, which incorporates a thicker (50–100 nm) metal film to protect cells from laser-related damage rather than a dynamic release layer as in traditional LIFT. This metallic layer is deposited by vacuum evaporation and interacts with the laser at the print

**FIGURE 4.1**

General schematic for laser-assisted bioprinting.

ribbon interface. As KrF excimer lasers ($\lambda = 248$ nm) used for AFA-LIFT have a penetration depth of 10–20 nm, the thick metal layer protects cells from UV irradiation effects. Depending on the laser wavelength, the pulse width at half max pulsing frequency can range from 500 fs to 30 ns. Femtosecond lasers impose more unfavorable mechanical force on the cells. In AFA-LIFT, the cell suspension medium, 140–160 μm thick, is spread onto the metal layer. Distance between the ribbon and receiving substrate is typically between 600 and 1000 μm . Spot size and fluence vary, but the apparatus described by [Hopp, Smausz, and Nógrádi \(2010\)](#), uses 250–300 μm diameter spot size and 200–350 mJ/cm^2 fluence. Thermal expansion of the metallic layer expels a volume of cell suspension to the receiving substrate rather than force generation via vaporization of the cell suspension medium. Some AFA-LIFT systems use charge-coupled device cameras to observe and record the transfer process and motorized stages to enhance laser focusing and cell placement ([Hopp et al., 2010](#)).

4.2.3 MATRIX-ASSISTED PULSED-LASER EVAPORATION DIRECT WRITE

MAPLE-DW facilitates forward transfer of biological materials in a controlled environment by utilizing two 3D motorized computer-controlled stages and automated features. Additionally,

MAPLE-DW is an all-optical imaging and printing system, with environmental controls in the current version. These are among the features that distinguish it from AFA-LIFT and other laser-assisted transfer systems. The current system is the culmination of improvements from previous generations; the first generation MAPLE-DW system had no motorized stages, the second generation added motorized stages and incorporated CAD/CAM features and the contemporary third-generation system has subsequently added environmental control. Previously, during printing, it has been difficult to keep small volumes of hydrated and dissociated cells alive for postprinting attachment. Environmental control, which includes control and monitoring of temperature and relative humidity, mitigates this without disrupting the print pattern or changing other print parameters.

Screen captures from the graphic user interface (GUI) of the third-generation MAPLE-DW system is shown in Fig. 4.2. This GUI enables researchers to control ribbon and substrate stage positioning, laser fluence, pulse trigger, local temperature and relative humidity. A live video feed of the print ribbon allows researchers to inspect and select cell groups for printing. The live video feed is shown on the right side of the first screen in Fig. 4.2. Geometric arrays, 2D patterns, and 3D constructs can be printed in a semiautonomous way owing to incorporated CAD and CAM tools. Cell selection, the rate-limiting step, still needs to be done manually, significantly decreasing the processing speed. In order to automate single-cell printing, “Machine Vision” (MV) capability was incorporated into the latest generation of MAPLE-DW. The MV image analysis algorithms identify and locate individual cells for transfer into user-defined patterns, thereby increasing the fabrication rate and achieving fully automated printing.

MAPLE-DW print ribbons are coated with a thick ($\sim 30\ \mu\text{m}$) sacrificial biopolymer layer, frequently Matrigel or gelatin, to absorb laser energy instead of the metal layer used in AFA-LIFT. Dissociated cells are partially encapsulated into the hydrogel layer, leaving a sacrificial zone between the ribbon-biopolymer interface and cell layer. MAPLE-DW uses low-powered lasers in UV or near-UV range, typically with a pulse duration of 8 ns. Single pulses focused at the quartz ribbon—absorption layer interface cause volatile bubble formation that enables transfer. Bubble formation and the beginning of material transfer are schematically represented in Fig. 4.3. Shallow penetration depth associated with low power UV lasers prevents direct effects and interaction between the cells and laser, preventing adverse effects on the cells (Riggs et al., 2011).

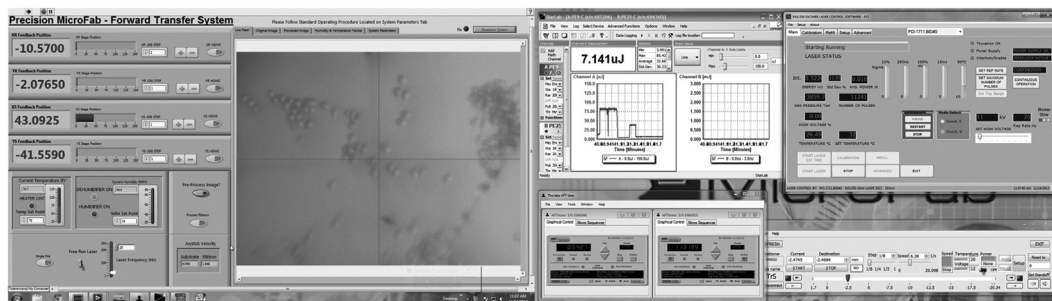
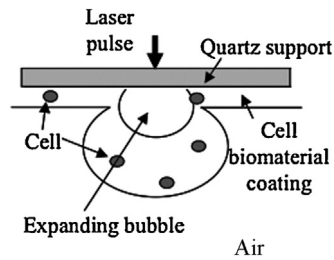


FIGURE 4.2

Dual screen GUI for MAPLE-DW.

**FIGURE 4.3**

Droplet formation and expanding bubble schematic (Wang et al., 2009).

4.2.4 ANCILLARY MATERIALS

Materials chosen for the print ribbon and receiving substrate can maintain cell viability before printing and promote cell proliferation, growth, migration and differentiation after printing. Poor choices for these mediums can lead to cell damage and death. In general, hydrogel biopolymers are used for print ribbons and substrates, but the amounts and biopolymer materials used should be carefully considered.

Cells are embedded in biopolymer hydrogel precursors on the print ribbon. The print ribbon requires a biopolymer that allows cell adherence and admits cell ejection with a single laser pulse. For nonfilm-assisted transfer methods, such as MAPLE-DW, this layer acts as a volatile sacrificial medium. Shear thinning and controllable viscosity are incorporated into new biopolymers for print applications because such qualities permit adjustments to compensate for altered printing parameters and while maintaining single pulse ejection. Less viscous biopolymers (e.g., hydrogels) reduce the propagation and magnitude of the stress wave generated, and thus subsequent cell damage, during printing (Wang, Li, & Huang, 2009).

Gelatin has been an effective biopolymer and is used for both film-assisted and nonfilm-assisted methods (Hopp et al., 2005; Ringeisen et al., 2004). Gelatin is amenable to thermal manipulation and can be partially cross-linked using heat, which reduces pressure on the embedded cells during printing. Thickness of this coating affects cell viability and varies based on the system and cells of interest. Ringeisen et al. (2004) found that cell viability increased from 50% to greater than 95% when their Matrigel biopolymer coating increased from 20 to 40 μm for pluripotent embryonal carcinoma cells transferred using the MAPLE-DW platform.

Biopolymers with various growth factors and/or extracellular matrix (ECM) components keep cells hydrated and promote cell adherence. However, growth factors and ECM can introduce additional variability and complications. ECM proteins may cause cells to bind firmly to the coated print ribbon, and thus require more power to achieve cell ejection and, in turn, cells are exposed to more irradiation and suffer greater eventual impact damage. Matrigel, one such material, is often a good choice of biopolymer and has been extensively utilized by laser DW researchers, even though it has been shown to have undesirable effects on cells, such as unintended stem cell differentiation (Riggs et al., 2011; Vukicevic et al., 1992).

The receiving substrate serves to cushion impact-induced stress, maintain hydration and provide the appropriate growth environment for posttransfer cells. Gelatin and Matrigel are both used as

receiving substrate biopolymers. Impact-induced stress and modeling are discussed in the mechanistic section of this chapter.

4.3 MATRIX-ASSISTED PULSED-LASER EVAPORATION DIRECT-WRITE MECHANISTICS

The cell transfer process in MAPLE-DW occurs in three sequential events: cellular droplet formation, cellular droplet travel, and cellular droplet landing. Analytical modeling of the two main events, cellular droplet formation and landing, and their effect on postdeposition cell viability are discussed in detail in the following section.

4.3.1 MODELING CELLULAR DROPLET FORMATION

External pressure and internal stresses are exerted on cells during rapid evaporation (normal boiling and phase explosion) and thermoelastic expansion of viscous droplets. The transformation of a superheated liquid to an equilibrium state of mixed phases is called a “phase explosion,” which eventually leads to a pressure pulse. The pressure pulses are systematically exploited by MAPLE-DW to generate printable droplets on demand. Although necessary for the formation of cellular droplets, the pressure pulse and thermoelastic expansion can injure printed cells, reducing viability after deposition. As such, both bubble formation and thermoelastic stress should be investigated via computational modeling to understand and minimize possible sources of damage to printed cells.

4.3.1.1 Modeling Bubble Formation-Induced Process Information

Figs. 4.3 and 4.4 are schematic representations of the laser-induced bubble formation and expansion process in a typical MAPLE-DW setup. While the MAPLE-DW scheme is shown here, the proposed modeling approach is applicable to other laser-based printing methods that utilize a sacrificial energy absorbing layer. The modeling assumes that the energy conversion thickness (<100 nm) at the ribbon-hydrogel interface is negligible.

During the bubble expansion process, a high-pressure pulse is generated, which ejects a droplet volume containing the cells. The bubble expansion process can be modeled using a computational domain as shown in Fig. 4.4. The materials involved consist of (1) vaporized gas bubble, (2) air,

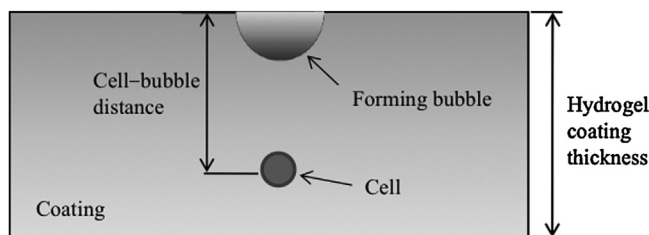


FIGURE 4.4

Modeling domain for the bubble expansion-induced cell deformation (Wang et al., 2009).

(3) hydrogel (used here as a coating material), and (4) the cell. Typically, the cell is modeled as a solid type material and applied a Lagrangian mesh for simplicity. The bubble, coating material, and air are modeled using the Eulerian mesh to avoid any extreme element distortion of these materials during ejection. The cell/hydrogel interaction is modeled using the appropriate Euler/Lagrange coupling to capture the effect of viscosity at the cell boundary layer. In addition, the interaction among the hydrogel, bubble gas, and air is modeled by defining the borders in a multimaterial grouping.

The pressure pulse accelerates resting cells from the ribbon until the droplet is ejected at a critical ejection velocity. Ejection velocity largely determines the initial velocity at which the cell droplet encounters the receiving substrate and should be controlled to minimize cell injury during landing. Fig. 4.5 shows the cell-center velocity evolution during the ejection process. It can be seen that the cell velocity oscillates initially and then gradually smoothens out to a constant ejection velocity, in this case, 107 m/s. This velocity oscillation is attributed to the elasticity of the cell, implying a negative acceleration. Due to the compressibility of the hydrogel, there is a delay in the velocity response to the bubble expansion as seen from Fig. 4.5. After approximately $2\ \mu\text{s}$, the cell droplet has a very weak connection with the coating material and starts to separate from the coating material at a constant velocity.

It has been found that the cell can initially accelerate as high as $10^9\ \text{m/s}^2$ at the beginning period of bubble expansion and then quickly approach zero in an oscillatory manner. Fortunately, this period of high acceleration is very brief, only lasting about $0.1\ \mu\text{s}$. The pressure that the cell experiences can also be very high at the beginning period of bubble expansion, but quickly decreases to

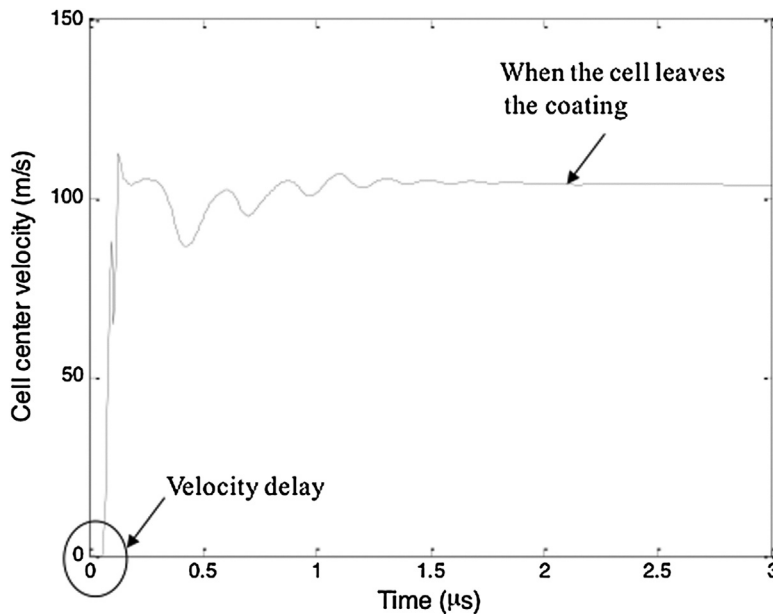


FIGURE 4.5

Cell-center velocity during the printing process (Wang et al., 2009).

zero in an oscillatory manner, as seen from the cell acceleration evolution. The top surface region of the cell usually experiences the highest pressure level, followed by the bottom surface and then middle regions (Wang et al., 2009).

4.3.1.2 Modeling Laser-Matter Interaction Induced Thermoelastic Stress

In general, and during cell deposition, localized heating and thermal expansion of a material cause thermoelastic stress. Two confinement conditions are necessary for the prominent generation of the thermoelastic stress: (1) the laser pulse duration should be much shorter than the characteristic thermal relaxation/diffusion time and (2) the laser pulse duration should also be shorter than the characteristic acoustic relaxation time to achieve a high-amplitude thermoelastic stress wave. If the laser beam size is taken as finite (laser spot diameter is comparable to the optical penetration depth), the wave generation becomes 3D, which can be solved analytically using Green's function. Unfortunately, this approach usually assumes the wave propagation is within a homogenous infinite medium. The image source method has also been explored to model this wave propagation challenge when one of the boundaries is rigid (Paltauf, Schmidt-Kloiber, & Frenz, 1998). However, the coating layer during MAPLE-DW is usually very thin. Consequently, this layer cannot be treated as an infinite medium as in a 2D case and the wave is reflected at the free surface. To better understand the effect of thermoelastic stress on the cell injury, the thermoelastic stress wave propagation is modeled here by considering the unique boundary conditions which are different from other previous studies, such as Paltauf et al. (1998).

As shown in Fig. 4.6, the computational domain used to simulate the thermoelastic stress generation is treated as 2D. This domain is due to the axisymmetric characteristic of the laser bioprinting process under a typical round laser spot. For the stress wave governing equation, the second-order central difference scheme is used to approximate spatial derivatives, and the backward difference scheme is used for the time derivative computation. The Crank–Nicolson method, which has second-order time accuracy, is used to solve the stress wave governing equation. For a finite thickness coating, the pressure wave reflection at the coating-air and the coating-glass interfaces has to be considered. Pressure reflection occurs at the coating-air and the coating-glass interfaces due to their acoustic impedances. The interface reflectivity is equal to -1 for a free surface (interface) and 1 for a rigid surface (interface). Thus the reflected stress at the rigid transparent support does not change the sign of stress due to the very high acoustic impedance in the rigid support, while it

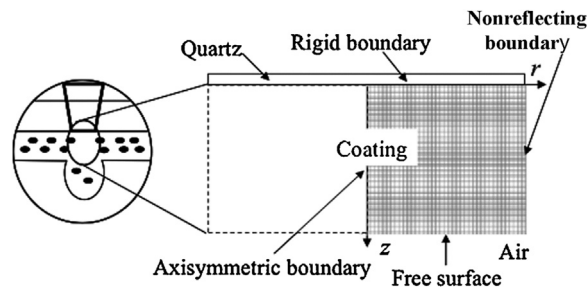


FIGURE 4.6

Schematic of the computational domain (Wang et al., 2009).

does change its sign due to the reflection at the free surface. The stress wave may be canceled by the reflected stress wave in the near vicinity of the coating-air free surface since the reflected stress wave has an opposite sign.

Fig. 4.7 shows the pressure profile at a fixed location, $50\text{ }\mu\text{m}$ below the laser spot center, for the first 140 ns (Wang, Lin, & Huang, 2011). It is found that a bipolar pressure pulse is developed. A bipolar pulse such as this was also observed in the study of the acoustic wave field generated in front of a submerged fiber tip by Paltauf et al. (1998). At about 33 ns after laser radiation, a positive compressive pressure arrives at this fixed location, immediately followed by negative tensile pressure. The first pressure peak (13.9 MPa magnitude) originates from the compressive pressure of

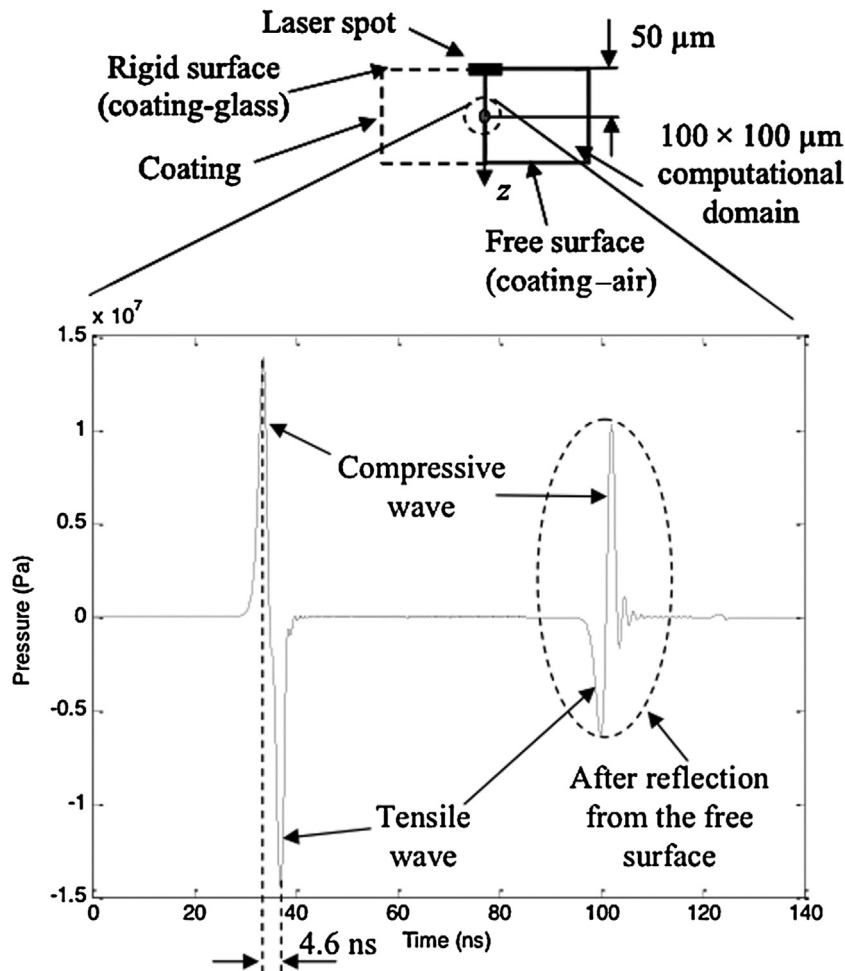


FIGURE 4.7

A representative pressure profile below the laser spot center ($z = 50\text{ }\mu\text{m}$) (Wang et al., 2011).

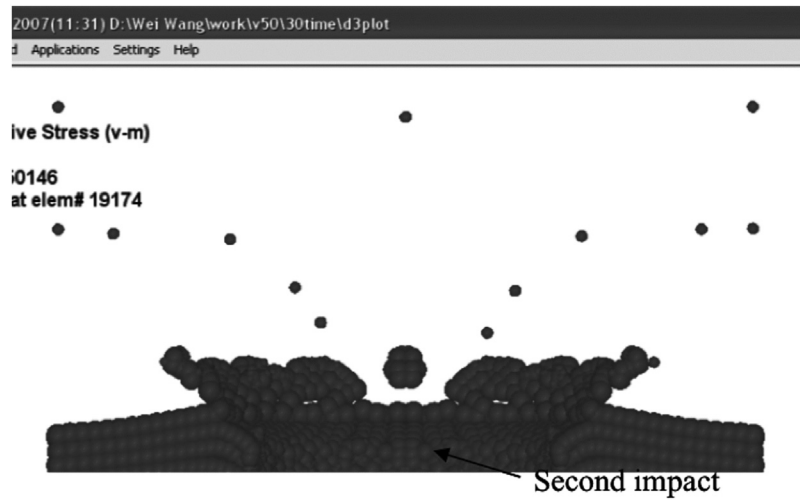
a plane wave, and the subsequent tensile pressure (-14.4 MPa magnitude) emits from the edge of the laser spot. Both compressive and tensile components physically coexist for the sake of the momentum conservation (Vogel & Venugopalan, 2003). They are experienced 4.6 ns apart on the order of 10 MPa at this fixed location, $50\text{ }\mu\text{m}$ below the center of the laser spot. At approximately 66 ns, the compressive pressure wave reaches the free surface and is reflected back into the coating medium as a tensile stress wave. Then, at approximately 100 ns, the first reflected wave reaches the fixed location with a peak magnitude of -6.4 MPa, and another compressive wave is observed with an even higher peak magnitude of 10.3 MPa; that is, a negative tensile pressure is followed by a larger positive compressive pressure. The second pressure pair is formed due to pressure reflection at the coating-air free surface, which changes the sign of pressure upon reflection. Since the wave energy is transmitted into the surrounding coating during propagation, both components of the second pressure pair decrease in magnitudes, as seen in Fig. 4.7.

4.3.2 MODELING OF DROPLET LANDING PROCESS

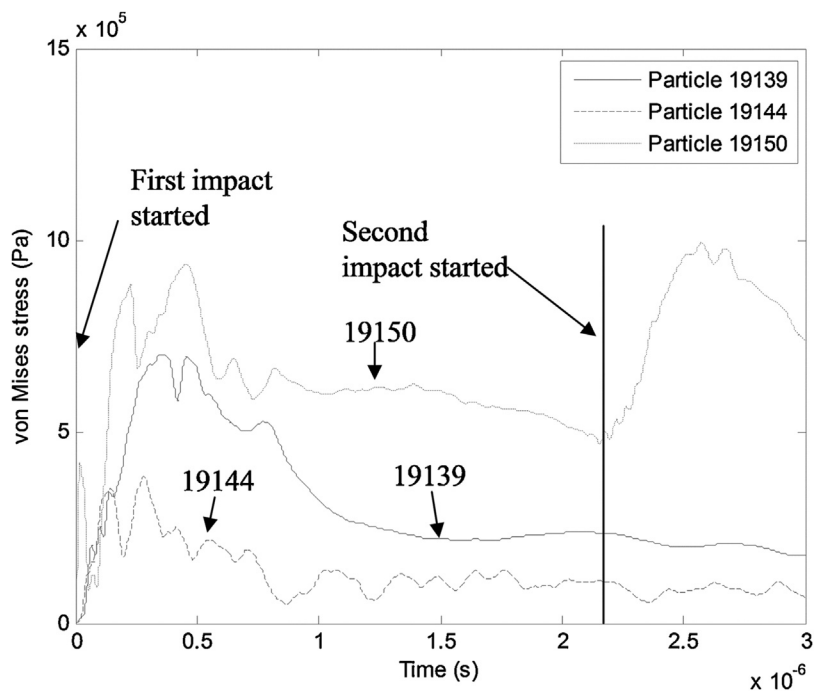
During landing, cell droplets undergo significant deceleration and impact(s), surviving a much higher external force than they are capable of surviving under steady state conditions. This landing process and its induced impact can be modeled using the mass, momentum and energy conservation equations (Wang et al., 2008; Wang, Huang, & Chrisey, 2007). These equations hold true for cells, hydrogel in the droplet, and the substrate coating. In addition to boundary and initial conditions, proper material models, which include the equation of state, constitutive model, and failure criteria, are also indispensable in solving these equations. The equation of state is used to define the functional relationship between pressure, density, and internal energy. The constitutive model defines the stress dependence of related strain, strain rate and temperature. Generally, a material model also includes a failure criterion to determine whether and when the material fails and loses its ability to support certain stress/strain.

A representative result of simulated landing is presented in Figs. 4.8 and 4.9 when a cell droplet with a velocity of 50 m/s hits a rigid substrate coated with a $30\text{ }\mu\text{m}$ thick layer of hydrogel. A cell droplet with a cell in the center is modeled using a mesh-free smooth particle hydrodynamic method. It can be seen that there are two different impacts during the process under the specified conditions. The first impact is between the cell droplet and the hydrogel coating, and the second impact is between the cell and the rigid substrate after the cell passes through the coating after the first impact. As the landing progresses, the hydrogel-enclosed cell droplet gradually merges into the substrate coating. After the cell is immersed in the coating (Fig. 4.8), the outside hydrogel enclosure and the coating bear relatively less stress even though the cell experiences more stress.

To study the von Mises stress and shear strain information during the landing process, three particles, the top particle 19,139, the inner particle 19,144 (one of the four center particles), and the bottom particle 19,150, are selected as the representative positions to better understand the overall cell response during the landing process. The simulation is performed using a coating thickness of $30\text{ }\mu\text{m}$ and an initial velocity (V_0) of 50 m/s. The particles' von Mises stress responses are shown in Fig. 4.9. The stress profiles show that two different impacts occur during the process under the specified conditions. First, impact happens at the computation start time, and the second impact happens at approximately $2.2\text{ }\mu\text{s}$. During the process, the peripheral particles 19,139 (top) and 19,150 (bottom) are subject to a higher stress level than the inner particle 19,144, which indicates that the cell membrane has a higher impact-induced mechanical stress during laser bioprinting. Also, the bottom particle

**FIGURE 4.8**

Landing process at 2.4865 μs .

**FIGURE 4.9**

Particle von Mises stress information (coating thickness = 30 μm and $V_0 = 50 \text{ m/s}$) (Wang et al., 2008).

19,150 undergoes a higher stress than the top particle 19,139. Fig. 4.9 also shows that the second impact has a negligible effect to the particles 19,139 (top) and 19,144 (inner). However, the bottom particle 19,150 experiences higher stresses during the second impact compared to the first impact (1.33 MPa vs 0.96 MPa), which means that it is of importance to study the stress information of the bottom particles during both the impacts. In this simulation, the bottom particle 19,150 experiences the first impact-induced stress peak at 0.2 μ s and the second peak at approximately 2.6 μ s. The probability of experiencing a second impact is highest for the bottom peripheral particles, lowest for the inner particles with the top peripheral particles falling somewhere in between.

Through modeling studies (Wang et al., 2007, 2008), it has been found that cell peripheral regions, especially the bottom peripheral region, usually experience a higher stress level than the inner regions. This indicates that the cell membrane can be adversely affected by the impact-induced mechanical injury during laser bioprinting. Additionally, the cell mechanical loading profile and the posttransfer cell viability depend on the cell droplet initial velocity and the substrate coating thickness. Generally, a larger initial velocity poses a higher probability of cell injury, and substrate coating can significantly relieve the cell mechanical injury severity. Furthermore, two important impact processes occur during the cell droplet landing process: first impact between the cell droplet and the substrate coating and second impact between the cell and the substrate. It is assumed that impact-induced cell injury depends on the magnitudes of stress, acceleration, and/or shear strain as well as the cell loading history. In fact, over the entire impact duration, the collective cell momentum change, rather than peak stress, acceleration and/or strain, appears to be critical in determining the cell viability during laser bioprinting and deposition.

4.4 POSTPROCESSING CELL VIABILITY AND FUNCTION

Postdeposition cell viability and biological functionality has been demonstrated for laser-assisted transfer techniques in terms of cell survival, adhesion, motility, proliferation, and differentiation. Damage to the cells can occur due to mechanical, thermal, or irradiative sources. Modeling of mechanical forces is detailed above. Due to the properties of the sacrificial hydrogel layer, thermal and irradiative injury is considered negligible. For example, in MAPLE-DW, for a print ribbon coating of 100 μ m, approximately 5 μ m depth would be affected by UV laser irradiation. In addition, thermal cell injury does not occur due to disparate time scales. During a typical 8–12 ns laser pulse, cell ejection from the print ribbon will occur within a few μ s (as previously shown) and heat conduction does not permeate through the first biopolymer layer to cause cell damage. As explained earlier in the discussion of ancillary materials, the choice of medium for both ribbon and substrate coating are crucial to cell viability (Lin et al., 2010; Riggs et al., 2011; Schiele et al., 2010).

Similarly, in a study on the transfer of “vulnerable cell types” via AFA-LIFT, Hopp et al. (2005) demonstrated successful transfer of rat Schwann and astroglial cells as well as pig lens epithelial cells. Two weeks after transfer, the cells survived, proliferated, differentiated and regained their original phenotypes. The three cell types were each isolated and then cultured in HEPES-buffered Dulbecco’s modified Eagle’s medium. Cells were then harvested from culture and prepared for AFA-LIFT printing. Cells were printed onto gelatin using an LLG two-pass amplification KrF excimer laser. Each target was irradiated with a single 30 ns pulse from the laser. Cell viability was inspected

using trypan blue dye, before and after printing, showed that 98%–99% of each cell type was alive before printing and 80%–85% of each type of the transferred cells remained alive after printing. In addition, the cells retained the ability to proliferate and differentiate 2 weeks after the initial printing.

Greater survival rates have been demonstrated with MAPLE-DW. Ringeisen et al. (2004) demonstrated a 95% cell survival rate when printing with pluripotent embryonic carcinoma cells. This MAPLE-DW array used an ArF excimer laser from Lambda Physik, model LPX 305, the wavelength of 193 nm, and 30 ns full width at half maximum. The target spot on the print ribbon was $100 \times 125 \mu\text{m}^2$ and incident laser fluence was in a controlled range from 100 to 500 mJ/cm². P19 (pluripotent embryonal carcinoma) cells were cultured and differentiated to neural and muscle cell phenotypes. Cells were prepared on the gelatin-coated ribbon. Printing was done in a humidity-controlled environment, as evaporation of droplet volume reduces cell survivability.

Cell viability was tested 6 hours following transfer using live/dead visibility kit. Cells printed onto a 40 μm layer of hydrogel resulted in greater than 95% posttransfer viability. Furthermore, the P19 cells retained their ability to differentiate in induction media.

This study also investigated DNA damage due to ultraviolet light exposure. Comet assays were performed to detect single and double-strand breaks. MAPLE-DW was used to perform noncontact cell transfer from the quartz ribbon directly into α -MEM. After comparison with control groups, there was no statistically significant damage to the cell DNA as a result of the ultraviolet laser interaction with the cell. This mitigation is due to the energy absorption by the sacrificial biopolymer on the quartz print ribbon.

With laser-assisted cell transfer methods, researchers benefit from soft cell deposition into programmable pattern positions to study cell behavior.

4.5 CASE STUDIES AND APPLICATIONS ILLUSTRATING THE IMPORTANCE OF SINGLE-CELL DEPOSITION

High-throughput, parallel analysis of single-cell behavior within the context of biological screening tools or structured cell–cell interaction interfaces necessitates the ability to isolate and position individual cells into engineered arrays. The rationale for intentional cell isolation is to identify significant singularities which may be lost when averaging response across an entire population of heterogeneous cells (Birtwistle et al., 2012). Resolving single-cell characteristics allows researchers to identify cells with the greatest differentiation potential—for example, pluripotent stem cells or cancer stem cells (Shackleton et al., 2006). Similarly, as tissues are composed of various cell types, the dynamics of cells from an explant or whole organism can drown out the finer nuances of the cell–cell interaction of interest. Re-integrating individual cells into a spatially controlled construct containing discrete voxel of single cells simplify the cellular cross talk. In this section, we explore case studies of single-cell patterns, organized by general application.

4.5.1 ISOLATED-NODE, SINGLE-CELL ARRAYS

The simplest single-cell arrays are ones wherein each cell is a standalone node for independent analysis. Neighboring cells, isolated by physical or spatial means, cause negligible effects on an individual

cell's response to presented external cues. These types of arrays are particularly useful for studying cell plasticity in response to external stimuli or as screening tools to identify unique cells in a heterogeneous population. In the following cases, cell isolation and immobilization may be done in parallel. The ability to perform subsequent analysis in a high-throughput manner is critical. By arranging cells into organized arrays, high-throughput analysis can be done in an efficient manner.

In cases where arrays are populated by clonally similar cells, studies focus on individual cell response to combinatorial external stimuli such as ECM, growth factors, and therapeutics. For example, the correlation of cell–matrix interactions to biomechanical transduction events caused by *in situ* stresses localized at the cellular boundaries benefits from single-cell resolving technologies. These fundamental studies utilize chemical micropatterning of surfaces to dually generate attachment islands of various geometries and present combinatorial ECM cues for assays that profile cell adhesion, morphology, and cytoskeletal stresses (Gallant, Michael, & García, 2005; Kuschel et al., 2006; Théry, 2010). In general, these differential-surface assays contain islands 2–20 μm in diameter to bind single cells. Recent work by Ma et al. (2013) constrained cells in 3D volumes by laser-guided micropatterning to study contractile functions of differentiated mesenchymal stem cells. Subsequent analysis, ranging from fluorescence image-based analysis of mechanical stress fibers to atomic force microscopy tip force probing, identified distinctive ECM components that may serve therapeutic targets in disease states.

Although static adhesion islands are suitable for cell–matrix adhesion assays, additional complexity is required for studies that investigate dynamic cell interaction with soluble cues in an external microenvironment, such as growth factors or therapeutic drugs. Microfluidic device-based assays provide the means to organize individual cells and directly interrogate them with a combinatorial library of soluble factors. High-throughput, single-cell resolving is achieved by physically catching cells in sized chambers against the flow stream. Because microfluidic platforms utilize flow rates in the laminar region (Reynold's number <1000), gradual mixing allows for continuous presentation of defined chemical gradients to cell arrays (Di Carlo, Wu, & Lee, 2006; Zheng et al., 2012a, 2012b).

Techniques that combine microfluidics and laser-based micropatterning have also been developed for heterotypic cell populations. An example of such an approach utilized novel microfluidic chips as vehicles for cell delivery and coupled it with a laser-guided cell micropatterning system to place individual cells in heterotypic 2D and 3D arrays. Reproducible cellular arrays of chick fore-brain neurons and glial cells were constructed with micron-level accuracy by trapping single cells at the outlets of microfluidic channels by pulsatile injections and using the laser to transfer them to target sites on gridded coverslips (2D substrate) and microfabricated polydimethylsiloxane “castles” with radial channels (3D substrate). Results showed that single cells could be patterned in as little as 20–30 seconds, attesting to the high throughput of laser-based techniques (Erdman et al., 2015).

To date, single-cell arrays have been used to monitor cellular-level response to growth, cytokine signaling, migratory, and metabolic factors (Cheng et al., 2010; Grecco et al., 2010; Köstler et al., 2013). As a natural extension and spin-off, such devices have also been used for drug screening and resistance studies (Kuss et al., 2013).

In cases where heterogeneous cell populations must be sorted, single-cell arrays provide ideal platforms for identification and subsequent processing. For example, adult stem and cancer stem cell populations consist of heterogeneous constituent cells. Some cells differentiate into new tissues while others are more likely to differentiate into metastatic lesions. Fractionation of such

heterogeneous populations down to the single-cell level elucidates the internal cellular mechanism that gives cells their unique phenotype. Nontrivial isolation and identification of pluripotent stem cells requires high-throughput genetic analysis to process clonally unique cells. Generally, microfluidic devices are used to capture single stem or cancer cells and allow for *in situ* lysing for genetic analysis (Czyż et al., 2014; Liang et al., 2013; Liu et al., 2013; Wilson et al., 2014; Wood et al., 2010).

4.5.2 NETWORK-LEVEL, SINGLE-CELL ARRAYS

Homotypic and heterotypic cell–cell communication dictate events that regulate tissue function, and disruption of this cellular signaling mechanisms leads to disease states (Pirlo et al., 2011). However, interrogation of desired cell–cell interactions may be hampered by background noise produced by extraneous cellular regulating events. Omission of these irrelevant processes and deposition of desired cells into a pattern drastically simplifies the nature of studied cross talk into well-defined, intentional reciprocal interactions. Unlike single-cell arrays in which each node is independent contact with the external environment (previous section), in network-level single-cell arrays, cells interface with each other and produce interdependent responses that cumulate in network-level behavior. Thus the two most important parameters are (1) identity of the neighboring cells, as the different cells illicit different interactions, and (2) distance between individual cells, as distance defines the mode of cellular communication (juxtacrine vs paracrine signaling). Laser DW technologies are particularly advantageous compared to conventional micropatterning techniques when it comes to dealing with cellular heterogeneity. Laser-assisted bioprinting offers the ability to incorporate and modulate multiple-cell types in the same array, which allows for quantitative probing of single-cell behavior as well as visualizing the interactions between multiple cells (D’Arcangelo & McGuigan, 2015).

Two research areas where single-cell arrays have demonstrated applicability are cancer invasion and neural networks.

Tumor invasion is a critical step in cancer progression to lethal metastatic disease and understanding the cancer-stroma interface will provide clues to new therapeutic targets. Stroma refers to the connective and supportive tissue in the tumor microenvironment. Fabricating artificial tissue interfaces at the single-cell level allows for the study of high-resolution cell–cell interactions at the micrometer length scale. Microfluidic and micropatterned devices have been fabricated to study both paracrine signaling (Hong, Pan, & Lee, 2012) and DC events (Frimat et al., 2011; Nikkhah et al., 2011; Zhang et al., 2014), but are limited to isolated pair-wise interactions. DW tools, such as laser-based bioprinters, are advantageous in depositing cells over a homogeneous area to study network-level behavior. Printing cells onto homogeneous substrates ensure that the development of the construct is influenced solely by guidance cues from neighboring cells, rather than limited by physical chambers or adhesion islands. In addition, there has been recent a push toward 3D tissue models to better study the *in vivo* cancer-stroma cross talk. Several researchers have demonstrated the patterning of single hydrogel microbeads containing cancer cells into small-scale patterns (Dolatshahi-Pirouz et al., 2014; Phamduy et al., 2012). Larger patterns have also been generated by coprinting 3D hydrogel biomaterials and cells (Gruene et al., 2011; Kingsley et al., 2013; Vinson et al., 2017).

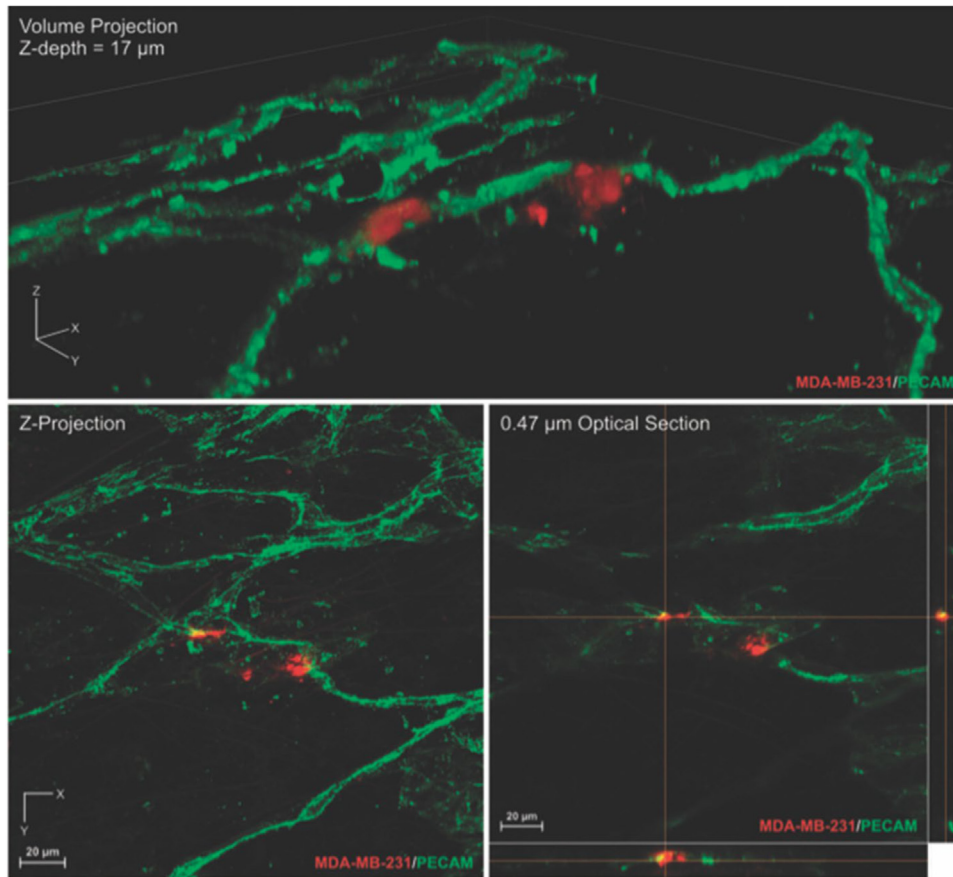
An additional unique and powerful attribute of laser DW bioprinting is the ability to print cells on virtually any substrate, including living tissue while preserving viability and biological function. As discussed above, the study of cancer-stroma interactions in the tumor microenvironment is an

area highly conducive to the precision of laser bioprinting and printing cancer cells on vascularized live tissue adds another dimension of physiological relevance for the study of cancer cell migration, invasion, and metastasis. MAPLE-DW has been used to print triple-negative breast cancer cells (MDA-MB-231) in spatially defined patterns onto vascularized mesentery tissue harvested from Wistar rats. The capabilities of MAPLE-DW, namely the CAD/CAM synchronization, *in situ* real-time imaging of the cell transfer process and high precision, allow for locating a desired spot on the substrate—in this case, a region in the proximity of a blood vessel—and patterning cells with single-cell resolution at that spot. Viable printed cancer cells (labeled in red) were observed using fluorescent confocal microscopy to actively interact with blood and lymphatic vessels (labeled in green) in the depth of the tissue, as depicted in Fig. 4.10. Cancer cells were also observed to migrate toward regions of high vascular density on the mesentery tissue—a phenomenon commonly observed *in vivo*. Moreover, the presence of cancer cells caused new blood vessel sprouting at the printing location, which recapitulates neo-angiogenesis frequently observed in the cancer microenvironment (Baldacchini et al., 2021; Burks et al., 2016; Phamduy et al., 2015). This application serves as a further attestation to the potential of laser bioprinting in dissecting cell dynamics in a metastatic cancer microenvironment.

In monoculture neural networks, guiding spontaneous synaptic connectivity requires defined spacing and pathways between individual cells. Several schemes have been developed toward this end. Dinh et al. (2013) generated high-density interconnected circuits of compartmentalized neurons in separate microfluidic chambers, with a biomaterials-guided outgrowth path between pairs of cells. Sanjana and Fuller (2004) and Macis et al. (2007) printed adhesion islands onto nonadherent substrates to allow for both cell attachment and directed outgrowth. In such examples, cell bodies are stationary and outgrowth occurs due to physical guides. Difato et al. (2011) utilized the laser-based optical tweezers technique to generate neural network patterns on homogeneous surfaces, which allows for cell migration in addition to synaptic development due to soluble guidance cues. Extracellular recording of action potential stimulation reveals that increasing internodal distance accelerates signal propagation (Wu et al., 2012), but decreases signal propagation efficiency (James et al., 2004). Single-neuron patterns with defined synaptic networks open up new avenues for fundamental biology studies and neural sensor/actuator applications.

4.5.3 NEXT-GENERATION SINGLE-CELL ARRAYS: INTEGRATED, COMPUTATION-DRIVEN ANALYSIS

As single-cell arrays are generally compared against a combinatorial library of soluble cues or first-neighbor cells, computational tools could be used to predict or validate empirical data in next-generation platforms. Standalone technology for high-throughput single-cell analysis currently exists. However, integration of existing technologies with single-cell arrays, especially in the context of network arrays, would produce a synergistic platform that is greater than the sum of its parts. Such improved platforms would provide *in situ*, multiplexed analysis of cell behavior and characteristics including morphology, biochemical state (Burguera, Bitar, & Bruinink, 2010), biomechanical stresses, gene expression (Vanneste et al., 2012), metabolism, and migration (Zheng et al., 2012a, 2012b). Information gleaned from arrays can then be compared to and contrasted with *in vivo* observations by computational analysis by compiling a database of single-cell array

**FIGURE 4.10**

Interactions between cancer cells and microvascular networks are shown. MDA-MB-231 cells were identified interacting and integrating with blood vessels using submicron confocal microscopy (Baldacchini et al., 2021; Burks et al., 2016; Phamduy et al., 2015).

responses and utilizing advanced machine learning algorithms to perform data analytics and pattern recognition. Striving for such unambiguous testing motivates research scientists to push the boundaries of precision bioprinting technologies.

4.5.4 EXAMPLE OF SINGLE-CELL ARRAY VIA MATRIX-ASSISTED PULSED-LASER EVAPORATION DIRECT WRITE

Fig. 4.11 is a 4×4 array pattern (800 μm center-to-center spacing) generated by MAPLE-DW and populated by single MDA-MB-231 human breast cancer cells. The phase-contrast image was taken immediately after the printing process, so that the transferred gelatin droplets appear raised on the

gelatin-coated substrate. In addition, each droplet contains single cells in their suspended state, which will eventually attach to the underlying substrate surface.

4.5.5 LASER DIRECT WRITE FOR NEURONS

The nervous system represents the most complicated organ system in the body, controlling all higher order faculties. As Tim Berners-Lee stated in 1999, “All that we know, all that we are, comes from the way our neurons are connected. Its fascinating complexity makes it one of the most difficult systems to study. Many advances have been made in studying population level events in the brain (e.g., fMRI, MEG, and PET), but mechanistic approaches to ascertaining fundamental neurobiology at the cellular level are limited, preventing a complete understanding of the governing order.”

As previously mentioned, the ability to study neuronal cell behavior in a deconvoluted manner has value to understanding both environmental and cell–cell interactions which regulate mammalian systems. Interest in such biological mechanisms spans the breadth of subjects from developmental neuroscience to functioning electrophysiology, with the added benefit of understanding and treating pathological disease states. Not until researchers can tease apart each component will we be able to truly appreciate how the nervous system’s complex pieces fit together. Currently, to examine neurons *in vitro*, the most commonly available methods involve organotypic slices or dissociated cell cultures. Studying individual neuronal characteristics within the context of a defined slice of living brain circuitry has led to some incredible insights, but the inability to parse out individual influences undermines the capacity to examine distinct mechanistic components (Gahwiler et al., 1997; Shankar et al., 2007; Yamaguchi et al., 2003). Dissociated cell cultures, on the other hand, offer more control over cell types and the influences of extracellular factors (Potter & DeMarse, 2001; Shahaf & Marom, 2001), while inherently negating the complex spatial organization present *in vivo*. While some control of cell – level interactions can be modulated through seeding density, the random nature of dissociated cultures limits a researcher’s ability to define specific cell–cell interactions in a contextual manner.

Some of the techniques to engineer both isolated node and network-level single-cell arrays were briefly described above. These technologies involve either “trapping” cells through patterned adhesion molecules or microfluidics, or “printing” cells through extrusion or inkjet deposition. Again, limitations currently exist on spatial accuracy and cell specificity, with study of nuanced interactions hinging on the ability to control the way distinct cell types interact. To that end, organized patterns of both 2D and 3D printed neuronal cells using laser DW has been demonstrated, with cells maintaining the ability to sprout axons (Curley et al., 2016; Patz et al., 2006). Laser DW addresses advantages for both shortcomings in a manner that has so far been underutilized for neural applications, despite the significant need.

4.5.5.1 Neural Development

Early development of the nervous system is a highly organized and multifaceted process in which environmental and extracellular inputs, including biochemical and mechanical cues, govern the emergence of increasingly differentiated cell types. The first experiment to show that cells influence the fate of other cells was performed in 1924 using a crude method to separate and grow developing neuronal cells (Spemann & Mangold, 2001). Since then, the use of bioprinting for

underlying substrate surface.

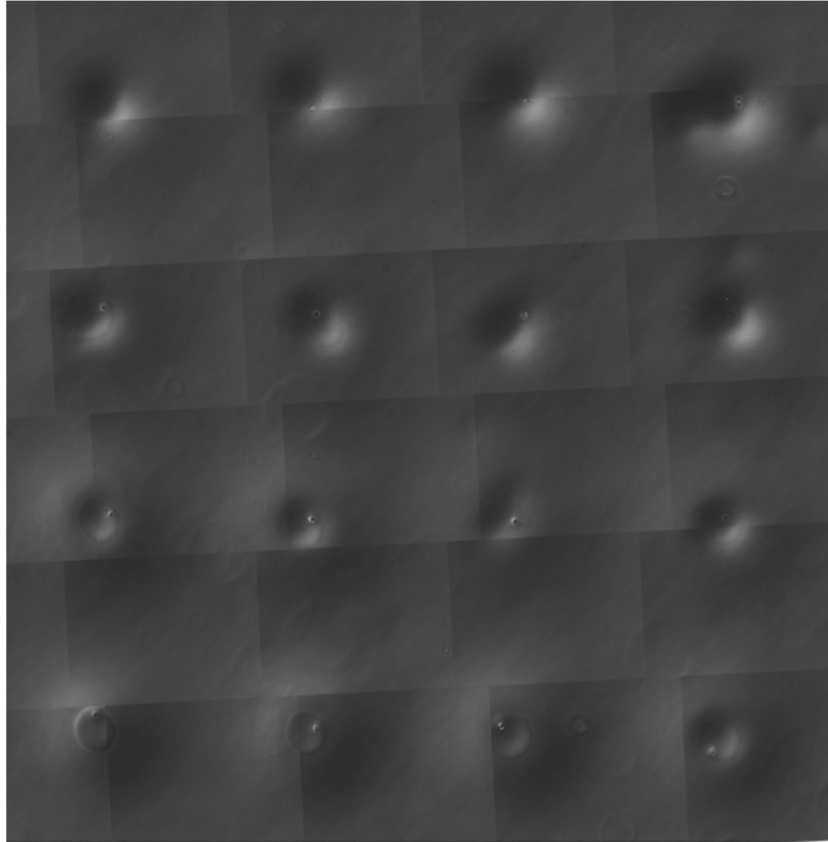


FIGURE 4.11

Single-cell array via LDW.

precise control of cell–environment interaction facilitated studies of neural stem cell differentiation into neurons, astrocytes and oligodendrocytes. Ilkhanizadeh et al. demonstrated that guidance of neural stem cell fate was possible through inkjet printing (Ilkhanizadeh, Teixeira, & Hermanson, 2007). Additionally, 3D printed microarrays of embryonic stem cells were able to characterize factors influencing neural commitment in a high-throughput capacity (Fernandes et al., 2010). Lastly, laser micromachining of growth substrates was shown to grow, proliferate, and differentiate neuroblasts in reproducible and controllable patterns (Doraiswamy et al., 2006). The combination of environmental manipulation with single-cell printing would allow combinatorial studies of cell–cell and cell–environment interactions on differentiation pathways.

Similarly, targeted guidance of neuronal processes to their proper functional targets is an amazingly precise and intricate process governed by attractive and repulsive signals. The sprouting axon of each developing neuron samples its surroundings and makes directional decisions through its

growth cone. By engineering growth substrates, an increased understanding of how axons interact with the extracellular environment has begun to develop, including how individual molecular guidance cues affect guidance (McCormick & Leipzig, 2012; Yu, Leipzig, & Shoichet, 2008). Single-cell printing can be uniquely valuable in recapitulating the numerous external cellular “guideposts” each axon encounters along its journey, to study the multitude of cellular interactions that coalesce during development.

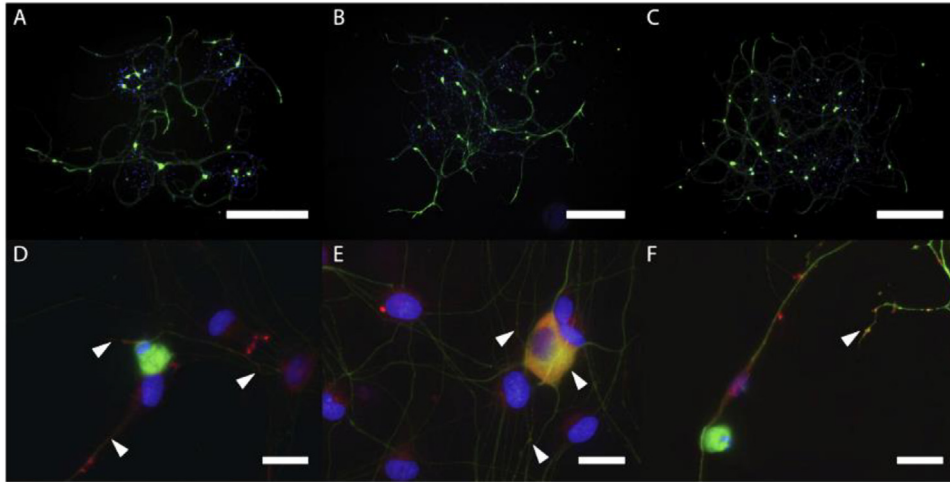
4.5.5.2 Engineered Circuits

The ability to fabricate highly specific patterns of neuronal cells has the potential to lend insight into many of the functional units of the brain and beyond. Xu et al. were the first to demonstrate that printed neuronal cells retained healthy electrophysiological characteristics (Xu et al., 2006). The subsequent ability to engineer neuronal circuits at the single-cell level was demonstrated by Edwards et al. with hippocampal neurons (Edwards et al., 2013). In a similar vein, techniques have been demonstrated to control individual synaptic connectivity of larger homogenous neuronal populations, enabling specific control over network and circuit properties (Staii et al., 2009; Vogt et al., 2005).

These methods do not allow for incorporation of multiple-cell types, severely limiting their ability to recapitulate critical heterogeneous cell synapses. The advantages of laser bioprinting to control the precise location and association of multiple-cell types, in combination with hydrogel and biomolecular based models for controlled axonal outgrowth, would allow the creation of mono and polysynaptic circuits between heterogeneous neuronal populations (Curley & Moore, 2011; Horn-Ranney et al., 2013). Laser DW represents the only technology currently available to accomplish this complicated feat.

Recently, Curley et al. demonstrated accurate and reproducible patterning of laser DW patterning of primary dorsal root ganglion (DRG) neurons and associated supportive cells maintaining cell viability and growth. Spinal columns were isolated from day 15 embryos of Long Evans rats, from which DRGs and supportive cells were isolated. MAPLE-DW was used to pattern neurons with a laser fluence of 0.4 J/cm^2 onto poly-L-lysine and laminin-coated substrates. DRGs, an extremely sensitive cell type, displayed nearly 85% viability postprinting, which compared favorably with the 89% viability observed with regular pipetting. DRGs also displayed the ability to form neural networks via outgrowth of neurites indicating preservation of biological function posttransfer. Furthermore, presynaptic expression of glutamate (neurotransmitter) transporters confirms the retention of cellular functionality. Fig. 4.12 depicts immunofluorescent images of printed DRGs, stained for neurite cytoskeletal protein β -III Tubulin (green) and presynaptic glutamate transporter VGLUT2 (red) as well as their colocalization (Curley et al., 2016). The ability to recreate neural tracts of virtually any system at the single and multicell level using laser DW bioprinting would allow for unprecedented experiments into the function of higher order activities such as memory, learning, cognition, locomotion, and pain.

Highly specific brain regions are known to integrate and communicate through transient synapses, facilitating the signaling which encodes and dictates all activity. Further complicating matters, these neuronal connections are constantly being strengthened, weakened or lost based on external outputs. For example, within the hippocampus, multiple-cell types are known to control memory and spatial navigation (Dickerson & Eichenbaum, 2010; Turrigiano, 2012). Similarly, thalamocortical circuits govern cognition, behavior, and consciousness, with clinical implications in autism,

**FIGURE 4.12**

Immunofluorescent images of developing networks. Neurite extension (β III Tubulin, green) and cell nuclei (DAPI, blue) for entirety of printed area after DIV3 (A), DIV5 (B), and DIV7 (C) are visualized and increased intranodal connectivity and cell migration is apparent with time (scale bar = 500 μ m). (D)–(F) Presynaptic expression of glutamate transporters (VGLUT2, red), neurite extension and cell nuclei visualized after DIV7 (scale bar = 20 μ m). Colocalization of VGLUT2 and β III Tubulin depicted with arrows. Neuronal coexpression with VGLUT2 was observed (E) (Curley et al., 2016).

schizophrenia, and attention deficit disorder (Buonanno, 2010; Sudhof, 2008). Sensorimotor neuron integration is another wide-ranging pathway that influences motor control and proprioception as well as all of the five classic senses (Goulding et al., 2002). The ability to understand and engineer highly specific sensorimotor circuits also has implications in advancing prosthetic design to interface with native tissue and restore near-natural function (Guo et al., 2012; Raspopovic et al., 2014). Another system with implication in limb prostheses is the neuromuscular junction. Das et al. have defined and explored *in vitro* models for synapse formation, though to date only in heterogeneous dissociated cultures (Das et al., 2007, 2010). In both cases, precisely how synaptic plasticity influences information processing is not yet understood. All of the aforementioned systems are highly complex and divergent across applications, but laser DW could be used to manipulate long-term studies of cellular, genetic, and molecular influences on individual synapse formation and maintenance. Due to the stochastic nature of these systems, the high-throughput potential of laser DW will be critical to increasing our understanding of the normal function and will inform the treatment of pathological states.

4.5.5.3 Nonneuronal Interactions

The consequences of studying engineered interactions between neuronal and nonneuronal cells also have application outside of a synaptic function. Though the number is still contested, there are at least as many nonneuronal as neuronal cells in the brain (Azevedo et al., 2009). Clearly, supportive

glial cells are critical to nervous system function, but the intricacies of their interactions are not well characterized. Schwann cell and oligodendrocyte myelination of axons is required for physiologically relevant conduction of nerve impulses, and astrocyte–neuron communication influences both neuronal differentiation and synapse formation (Sherman & Brophy, 2005; Sofroniew & Vinters, 2010). Mirroring the sentiments of previous applications, glial cells are both necessary for natural neuronal function and responsible for many pathogenic states. Implications in Alzheimer’s, Parkinson’s, multiple sclerosis, neuropathy, and chronic pain have been tied to aberrant glial function (Antony, 2014; Block, Zecca, & Hong, 2007; Luongo, Maione, & Di Marzo, 2014; McMahon & Malcangio, 2009). In order to study such interactions, Park et al. constructed a microfluidic device to isolate axonal processes and enable coculture with both oligodendrocyte precursor and astrocyte cells (Park et al., 2012). They then saw evidence of healthy oligodendrocyte differentiation and myelination, alongside induced astrocytic and biomolecular damage. Goudriaan et al. also demonstrated the reciprocal nature of genetic disturbances in astrocyte–neuron interactions through a novel cellular isolation method (Goudriaan et al., 2014). Both tools represent powerful examples of potentially high-throughput studies in neuron–glia interaction. However, the speed and spatial resolution of laser DW and CAD/CAM technology would allow for the rapid manipulation of multiple-cell juxtaposition to define mechanisms for axon–glia communication and interaction. The combination of throughput, flexibility and accuracy would enable experiments to define and explore contact and signaling related influences toward understanding both healthy and pathological states.

4.5.5.4 Outlook

Through the examples given, it is clear that advances in technology have fundamentally increased our understanding of the nervous system. By incrementally deconstructing the intricate milieu of factors that govern the organization of the brain, both from a developmental and functional standpoint, researchers are beginning to recognize and understand the consequences of specific biological mechanisms. Engineered models utilizing laser DW techniques would enable the manipulation of molecular, cellular and synaptic contributions from a single-cell level, representing the next advancement required to decipher implications on emergent behavior in healthy and diseased states as well as inform potential treatment strategies.

4.6 Conclusion

Recent advances have led to laser bioprinting being regarded as a proven method for biomaterial transfer, with it being the only bioprinting approach truly capable of single-cell resolution. Owing to the unique combination of optical imaging, CAD/CAM control, researchers have created a platform capable of fabricating complex constructs from the bottom up with the potential for high-throughput fabrication of physiologically relevant biological constructs, that is, reproducible tissue constructs to the single-cell level resolution to test different drugs with iterative composition and/or spacing manipulations allowing for unambiguous conclusions about the influence of the cellular microenvironment. The MAPLE-DW platform, especially, has demonstrated greater than 5 μm spatial precision with posttransfer cell viability consistently greater than 90%. Researchers have achieved viable transfers with disparate cell types, thereby enabling an even greater number of

applications. Moreover, the short time required to make constructs coupled with conventional cell culture protocol and ease of printing make laser bioprinting with MAPLE-DW an efficient, feasible and reliable way to isolate and study cellular interactions and behaviors. *In vitro* and *ex vivo* experimental models created by laser DW for cancer, stem cell, and neural studies show promise for the future and are a disruptive technology in tissue engineering.

Some of the major challenges facing bioprinting and tissue engineering, which must be addressed in the next generation of bioprinting systems include further optimization of fabrication protocols, scaling up and advancing imaging capabilities. Laser-assisted single-cell printing enables more precise and reproducible studies, yet also hinders larger-scale tissue engineering until comprehensive pattern recognition and automation is realized. Next-generation laser-assisted bioprinters will have both single-cell print resolution as well as demonstrate parallelization to show that scaling up these systems is possible and eventually can be used for large scale tissue engineering in addition to clonal studies.

Improved imaging techniques will not only help researchers monitor and analyze deposition *in situ*, but also will be incorporated into automated data mining systems which will track deposition successes and failures and will associate each transfer attempt with printing metadata. Bioprinting metadata will include print conditions (e.g., temperature and humidity) and parameters (e.g., laser fluence and pulse duration) and be stored in a database with information on cell phenotypes, transfer precision and morphological changes. Using data analysis, mining, and visualization techniques, users will be able to learn from each cell transfer, accelerate system improvements and incorporate embedded systems and construct statistics in subsequent studies with the cell constructs.

Next-generation laser bioprinting systems need to build on current success and *continue* to look to other disciplines for inspiration. This approach will help push single-cell laser-assisted bioprinting to a point of enhanced functionality, reproducibility and parallelization. Single-cell printing is crucial but needs to be done with a systems-engineering approach that has an eye turned toward future applications and problems, such as the challenge of ensuring vascularization of tissue-engineered constructs to accelerate translation to the clinic. The MAPLE-DW platform advances this effort beyond other currently available technologies and demonstrates how generations of laser bioprinting systems evolve as a cutting edge technology that enables biomedical research.

References

- Antony, J. M. (2014). Neuropathogenesis: Rogue glia cause mayhem in the brain. *Translational Neuroscience*, 5(1), 91–98. Available from <https://doi.org/10.2478/s13380-014-0211-0>.
- Azevedo, F. A. C., et al. (2009). Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. *Journal of Comparative Neurology*, 513(5), 532–541. Available from <https://doi.org/10.1002/Cne.21974>.
- Baldacchini, T., Saksena, J., Sklare, S. C., Vinson, B. T., Huang, Y., Chrisey, D. B., & Narayan, R. J. (2021). Translation of laser-based three-dimensional printing technologies. *MRS Bulletin*, 46(2), 174–185. Available from <https://doi.org/10.1557/s43577-021-00042-2>.
- Barron, J. A., Krizman, D. B., & Ringeisen, B. R. (2005). Laser printing of single cells: Statistical analysis, cell viability, and stress. *Annals of Biomedical Engineering*, 33(2), 121–130. Available from <https://doi.org/10.1007/s10439-005-8971-x>.

- Birtwistle, M. R., Rauch, J., Kiyatkin, A., Aksamitiene, E., Dobrzyński, M., Hoek, J. B., Kolch, W., ... Kholodenko, B. N. (2012). Emergence of bimodal cell population responses from the interplay between analog single-cell signaling and protein expression noise. *BMC Systems Biology*, 6(1), 109. Available from <https://doi.org/10.1186/1752-0509-6-109>.
- Block, M. L., Zecca, L., & Hong, J. S. (2007). Microglia-mediated neurotoxicity: Uncovering the molecular mechanisms. *Nature Reviews Neuroscience*, 8(1), 57–69. Available from <https://doi.org/10.1038/Nrn2038>.
- Buonanno, A. (2010). The neuregulin signaling pathway and schizophrenia: From genes to synapses and neural circuits. *Brain Research Bulletin*, 83(3–4), 122–131. Available from <https://doi.org/10.1016/j.brainresbull.2010.07.012>.
- Burguera, E. F., Bitar, M., & Bruinink, A. (2010). Novel *in vitro* co-culture methodology to investigate heterotypic cell-cell interactions. *European Cells & Materials*, 19, 166–179.
- Burks, H. E., Phamduy, T. B., Azimi, M. S., Saksena, J., Burow, M. E., Collins-Burow, B. M., ... Murfee, W. L. (2016). Laser direct-write onto live tissues: A novel model for studying cancer cell migration. *Journal of Cellular Physiology*, 231(11), 2333–2338. Available from <https://doi.org/10.1002/jcp.25363>.
- Cheng, W., Klauke, N., Smith, G., & Cooper, J. M. (2010). Microfluidic cell arrays for metabolic monitoring of stimulated cardiomyocytes. *Electrophoresis*, 31(8), 1405–1413. Available from <https://doi.org/10.1002/elps.200900579>.
- Curley, J. L., & Moore, M. J. (2011). Facile micropatterning of dual hydrogel systems for 3D models of neurite outgrowth. *Journal of Biomedical Materials Research Part A*, 99(4), 532–543. Available from <https://doi.org/10.1002/jbm.a.33195>.
- Curley, J. L., Sklare, S. C., Bowser, D. A., Saksena, J., Moore, M. J., & Chrisey, D. B. (2016). Isolated node engineering of neuronal systems using laser direct write. *Biofabrication*, 8(1). Available from <https://doi.org/10.1088/1758-5090/8/1/015013>.
- Czyż, Z. T., et al. (2014). Reliable single cell array CGH for clinical samples. *PLoS One*, 9(1), e85907. Available from <https://doi.org/10.1371/journal.pone.0085907>.
- D’Arcangelo, E., & McGuigan, A. P. (2015). Micropatterning strategies to engineer controlled cell and tissue architecture *in vitro*. *Biotechniques*, 58(1), 13–23. Available from <https://doi.org/10.2144/000114245>.
- Das, M., et al. (2007). Embryonic motoneuron-skeletal muscle co-culture in a defined system. *Neuroscience*, 146(2), 481–488. Available from <https://doi.org/10.1016/j.neuroscience.2007.01.068>.
- Das, M., et al. (2010). A defined long-term *in vitro* tissue engineered model of neuromuscular junctions. *Biomaterials*, 31(18), 4880–4888. Available from <https://doi.org/10.1016/j.biomaterials.2010.02.055>.
- Di Carlo, D., Wu, L. Y., & Lee, L. P. (2006). Dynamic single cell culture array. *Lab on a Chip*, 6(11), 1445–1449. Available from <https://doi.org/10.1039/B605937F>.
- Dickerson, B. C., & Eichenbaum, H. (2010). The episodic memory system: Neurocircuitry and disorders. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, 35(1), 86–104. Available from <https://doi.org/10.1038/Npp.2009.126>.
- Difato, F., et al. (2011). Combined optical tweezers and laser dissector for controlled ablation of functional connections in neural networks. *Journal of Biomedical Optics*, 16(5), 051306. Available from <https://doi.org/10.1117/1.3560268>.
- Dinh, N.-D., et al. (2013). Microfluidic construction of minimalistic neuronal co-cultures. *Lab on a Chip*, 13(7), 1402–1412. Available from <https://doi.org/10.1039/c3lc41224e>.
- Dolatshahi-Pirouz, A., et al. (2014). A combinatorial cell-laden gel microarray for inducing osteogenic differentiation of human mesenchymal stem cells. *Scientific reports*, 4, 3896. Available from <https://doi.org/10.1038/srep03896>.
- Doraiswamy, A., et al. (2006). Two-dimensional differential adherence of neuroblasts in laser micromachined CAD/CAM agarose channels. *Applied Surface Science*, 252, 4748–4753. Available from <https://doi.org/10.1016/j.apsusc.2005.07.158>.

- Edwards, D., et al. (2013). Two cell circuits of oriented adult hippocampal neurons on self-assembled monolayers for use in the study of neuronal communication in a defined system. *ACS Chemical Neuroscience*, 4(8), 1174–1182. Available from <https://doi.org/10.1021/Cn300206k>.
- Erdman, N., et al. (2015). Microfluidics-based laser guided cell-micropatterning system. *Biofabrication*, 33(4), 395–401. Available from <https://doi.org/10.1088/1758-5082/6/3/035025.Microfluidics-Based>.
- Fernandes, T. G., et al. (2010). Three-dimensional cell culture microarray for high-throughput studies of stem cell fate. *Biotechnology and Bioengineering*, 106(1), 106–118. Available from <https://doi.org/10.1002/Bit.22661>.
- Frimat, J.-P., et al. (2011). A microfluidic array with cellular valving for single cell co-culture. *Lab on a Chip*, 11(2), 231–237. Available from <https://doi.org/10.1039/c0lc00172d>.
- Gahwiler, B. H., et al. (1997). Organotypic slice cultures: A technique has come of age. *Trends in Neurosciences*, 20(10), 471–477. Available from [https://doi.org/10.1016/S0166-2236\(97\)01122-3](https://doi.org/10.1016/S0166-2236(97)01122-3).
- Gallant, N., Michael, K., & García, A. (2005). Cell adhesion strengthening: Contributions of adhesive area, integrin binding, and focal adhesion assembly. *Molecular Biology of the Cell*, 16, 4329–4340. Available from <https://doi.org/10.1091/mbc.E05>.
- Goudriaan, A., et al. (2014). Novel cell separation method for molecular analysis of neuron-astrocyte co-cultures. *Frontiers in Cellular Neuroscience*, 8. Available from <https://doi.org/10.3389/Fncel.2014.00012>.
- Goulding, M., et al. (2002). The formation of sensorimotor circuits. *Current Opinion in Neurobiology*, 12(5), 508–515. Available from [https://doi.org/10.1016/S0959-4388\(02\)00371-9](https://doi.org/10.1016/S0959-4388(02)00371-9).
- Grecco, H. E., et al. (2010). *In situ* analysis of tyrosine phosphorylation networks by FLIM on cell arrays. *Nature Methods*, 7(6), 467–472. Available from <https://doi.org/10.1038/nmeth.1458>.
- Gruene, M., et al. (2011). Laser printing of three-dimensional multicellular arrays for studies of cell–cell and cell–environment interactions. *Tissue Engineering. Part C, Methods*, 17(10), 973–982. Available from <https://doi.org/10.1089/ten.TEC.2011.0185>.
- Guo, X. F., et al. (2012). Tissue engineering the monosynaptic circuit of the stretch reflex arc with co-culture of embryonic motoneurons and proprioceptive sensory neurons. *Biomaterials*, 33(23), 5723–5731. Available from <https://doi.org/10.1016/j.biomaterials.2012.04.042>.
- Hong, S., Pan, Q., & Lee, L. P. (2012). Single-cell level co-culture platform for intercellular communication. *Integrative Biology*, 4(4), 374–380. Available from <https://doi.org/10.1039/C2IB00166G>.
- Hopp, B., et al. (2005). Survival and proliferative ability of various living cell types after laser-induced forward transfer. *Tissue Engineering*, 11(11–12), 1817–1823. Available from <https://doi.org/10.1089/ten.2005.11.1817>.
- Hopp, B., Smausz, T., & Nógrádi, A. (2010). Absorbing-film assisted laser induced forward transfer of sensitive biological subjects. In B. R. Ringeisen, B. J. Spargo, & P. K. Wu (Eds.), *Cell and organ printing SE-7* (pp. 115–134). Springer. Available from http://doi.org/10.1007/978-90-481-9145-1_7.
- Horn-Ranney, E. L., et al. (2013). Structural and molecular micropatterning of dual hydrogel constructs for neural growth models using photochemical strategies. *Biomedical Microdevices*, 15(1), 49–61. Available from <https://doi.org/10.1007/s10544-012-9687-y>.
- Ilkhanizadeh, S., Teixeira, A. I., & Hermanson, O. (2007). Inkjet printing of macromolecules on hydrogels to steer neural stem cell differentiation. *Biomaterials*, 28(27), 3936–3943. Available from <https://doi.org/10.1016/j.biomaterials.2007.05.018>.
- James, C. D., et al. (2004). Extracellular recordings from patterned neuronal networks using planar microelectrode arrays. *IEEE Transactions on Bio-Medical Engineering*, 51(9), 1640–1648. Available from <https://doi.org/10.1109/TBME.2004.827252>.
- Kingsley, D. M., et al. (2013). Single-step laser-based fabrication and patterning of cell-encapsulated alginate microbeads. *Biofabrication*, 5(4), 045006. Available from <https://doi.org/10.1088/1758-5082/5/4/045006>.
- Köstler, W. J., et al. (2013). Epidermal growth-factor-induced transcript isoform variation drives mammary cell migration. *PLoS One*, 8(12), e80566. Available from <https://doi.org/10.1371/journal.pone.0080566>.

- Kuschel, C., et al. (2006). Cell adhesion profiling using extracellular matrix protein microarrays. *Biotechniques*, 40(4), 523–531. Available from <https://doi.org/10.2144/000112134>.
- Kuss, S., et al. (2013). Assessment of multidrug resistance on cell coculture patterns using scanning electrochemical microscopy. *Proceedings of the National Academy of Sciences of the United States of America*, 110(23), 9249–9254. Available from <https://doi.org/10.1073/pnas.1214809110>.
- Lewinski, N., Colvin, V., & Drezek, R. (2008). Cytotoxicity of nanoparticles. *Small (Weinheim an der Bergstrasse, Germany)*, 4(1), 26–49. Available from <https://doi.org/10.1002/sml.200700595>.
- Liang, P., et al. (2013). Drug screening using a library of human induced pluripotent stem cell-derived cardiomyocytes reveals disease-specific patterns of cardiotoxicity. *Circulation*, 127(16), 1677–1691. Available from <https://doi.org/10.1161/CIRCULATIONAHA.113.001883>.
- Liberski, A. R., Delaney, J. T., & Schubert, U. S. (2011). “One cell-one well”: A new approach to inkjet printing single cell microarrays. *ACS Combinatorial Science*, 13(2), 190–195. Available from <https://doi.org/10.1021/co100061c>.
- Lin, Y., et al. (2010). Effect of laser fluence in laser-assisted direct writing of human colon cancer cell. *Rapid Prototyping Journal*, 16(3), 202–208. Available from <https://doi.org/10.1108/13552541011034870>.
- Liu, Y., et al. (2013). Development of a single-cell array for large-scale DNA fluorescence *in situ* hybridization. *Lab on a Chip*, 13(7), 1316–1324. Available from <https://doi.org/10.1039/c2lc40364a>.
- Luongo, L., Maione, S., & Di Marzo, V. (2014). Endocannabinoids and neuropathic pain: Focus on neuron-glia and endocannabinoid-neurotrophin interactions. *European Journal of Neuroscience*, 39(3), 401–408. Available from <https://doi.org/10.1111/Ejn.12440>.
- Ma, Z., et al. (2013). Laser patterning for the study of MSC cardiogenic differentiation at the single-cell level. *Light, Science & Applications*, 2. Available from <https://doi.org/10.1038/lsa.2013.24>.
- Macis, E., et al. (2007). An automated microdrop delivery system for neuronal network patterning on micro-electrode arrays. *Journal of Neuroscience Methods*, 161(1), 88–95. Available from <https://doi.org/10.1016/j.jneumeth.2006.10.015>.
- McCormick, A. M., & Leipzig, N. D. (2012). Neural regenerative strategies incorporating biomolecular axon guidance signals. *Annals of Biomedical Engineering*, 40(3), 578–597. Available from <https://doi.org/10.1007/s10439-011-0505-0>.
- McMahon, S. B., & Malcangio, M. (2009). Current challenges in glia-pain biology. *Neuron*, 64(1), 46–54. Available from <https://doi.org/10.1016/j.neuron.2009.09.033>.
- Nikkhah, M., et al. (2011). MCF10A and MDA-MB-231 human breast basal epithelial cell co-culture in silicon micro-arrays. *Biomaterials*, 32(30), 7625–7632. Available from <https://doi.org/10.1016/j.biomaterials.2011.06.041>.
- Paltauf, G., Schmidt-Kloiber, H., & Frenz, M. (1998). Photoacoustic waves excited in liquids by fiber-transmitted laser pulses. *The Journal of the Acoustical Society of America*, 104(2), 890–897.
- Park, J., et al. (2012). Multi-compartment neuron-glia co-culture platform for localized CNS axon-glia interaction study. *Lab on a Chip*, 12(18), 3296–3304. Available from <https://doi.org/10.1039/C2lc40303j>.
- Patz, T. M., et al. (2006). Three-dimensional direct writing of B35 neuronal cells. *Journal of Biomedical Materials Research Part B-Applied Biomaterials*, 78B(1), 124–130. Available from <https://doi.org/10.1002/Jbm.B.30473>.
- Phamduy, T., et al. (2015). Printing cancer cells into intact microvascular networks: A model for investigating cancer cell dynamics during angiogenesis. *Intergrative Biology*, 7, 1068–1074.
- Phamduy, T. B., et al. (2012). Laser direct-write of single microbeads into spatially-ordered patterns. *Biofabrication*, 4(2), 025006. Available from <https://doi.org/10.1088/1758-5082/4/2/025006>.
- Phamduy, T. B., Corr, D. T., & Chrisey, D. B. (2010). *Bioprinting*. *Encyclopedia of industrial biotechnology* (pp. 1–18). Hoboken, NJ: John Wiley & Sons, Inc. Available from <http://doi.org/10.1002/9780470054581.eib131>.

- Piqué, A., Chrisey, D., & Auyeung, R. (1999). A novel laser transfer process for direct writing of electronic and sensor materials. *Applied Physics A*, 284, 279–284.
- Pirlo, R. K., et al. (2011). Laser-guided cell micropatterning system. *The Review of Scientific Instruments*, 82(1), 013708. Available from <https://doi.org/10.1063/1.3529919>.
- Potter, S. M., & DeMarse, T. B. (2001). A new approach to neural cell culture for long-term studies. *Journal of Neuroscience Methods*, 110(1–2), 17–24. Available from [https://doi.org/10.1016/S0165-0270\(01\)00412-5](https://doi.org/10.1016/S0165-0270(01)00412-5).
- Raspopovic, S., et al. (2014). Restoring natural sensory feedback in real-time bidirectional hand prostheses. *Science Translational Medicine*, 6(222), 222ra19. Available from <https://doi.org/10.1126/scitranslmed.3006820>, 2014/02/07.
- Riggs, B. C., et al. (2011). Matrix-assisted pulsed laser methods for biofabrication. *MRS Bulletin*, 36(12), 1043–1050. Available from <https://doi.org/10.1557/mrs.2011.276>.
- Ringeisen, B. R., et al. (2004). Laser printing of pluripotent embryonal carcinoma cells. *Tissue Engineering*, 10(3–4), 483–491. Available from <https://doi.org/10.1089/107632704323061843>.
- Sanjana, N. E., & Fuller, S. B. (2004). A fast flexible ink-jet printing method for patterning dissociated neurons in culture. *Journal of Neuroscience Methods*, 136(2), 151–163. Available from <https://doi.org/10.1016/j.jneumeth.2004.01.011>.
- Schiele, N. R., et al. (2010). Laser-based direct-write techniques for cell printing. *Biofabrication*, 2(3), 37–54. Available from <https://doi.org/10.1016/bs.mcb.2015.01.016.Observing>.
- Schiele, N. R., Chrisey, D. B., & Corr, D. T. (2011). Gelatin-based laser direct-write technique for the precise spatial patterning of cells. *Tissue Engineering. Part C, Methods*, 17(3), 289–298. Available from <https://doi.org/10.1089/ten.tec.2010.0442>.
- Shackleton, M., et al. (2006). Generation of a functional mammary gland from a single stem cell. *Nature*, 439(7072), 84–88. Available from <https://doi.org/10.1038/nature04372>.
- Shahaf, G., & Marom, S. (2001). Learning in networks of cortical neurons. *Journal of Neuroscience*, 21(22), 8782–8788.
- Shankar, G. M., et al. (2007). Natural oligomers of the Alzheimer amyloid-beta protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. *Journal of Neuroscience*, 27(11), 2866–2875. Available from <https://doi.org/10.1523/Jneurosci.4970-06.2007>.
- Sherman, D. L., & Brophy, P. J. (2005). Mechanisms of axon ensheathment and myelin growth. *Nature Reviews. Neuroscience*, 6(9), 683–690. Available from <https://doi.org/10.1038/Nrn1743>.
- Smausz, T., et al. (2006). Study on metal microparticle content of the material transferred with Absorbing Film Assisted Laser Induced Forward Transfer when using silver absorbing layer. *Applied Surface Science*, 252(13), 4738–4742. Available from <https://doi.org/10.1016/j.apsusc.2005.07.115>.
- Sofroniew, M. V., & Vinters, H. V. (2010). Astrocytes: Biology and pathology. *Acta Neuropathologica*, 119(1), 7–35. Available from <https://doi.org/10.1007/s00401-009-0619-8>.
- Spemann, H., & Mangold, H. (2001). Induction of embryonic primordia by implantation of organizers from a different species. 1923. *The International Journal of Developmental Biology*, 45(1), 13–38.
- Staii, C., et al. (2009). Positioning and guidance of neurons on gold surfaces by directed assembly of proteins using Atomic Force Microscopy. *Biomaterials*, 30(20), 3397–3404. Available from <https://doi.org/10.1016/j.biomaterials.2009.03.027>.
- Sudhof, T. C. (2008). Neuroligins and neuroligins link synaptic function to cognitive disease. *Nature*, 455(7215), 903–911. Available from <https://doi.org/10.1038/Nature07456>.
- Théry, M. (2010). Micropatterning as a tool to decipher cell morphogenesis and functions. *Journal of Cell Science*, 123(Pt 24), 4201–4213. Available from <https://doi.org/10.1242/jcs.075150>.
- Turrigiano, G. (2012). Homeostatic synaptic plasticity: Local and global mechanisms for stabilizing neuronal function. *Cold Spring Harbor Perspectives in Biology*, 4(1). Available from <https://doi.org/10.1101/cshperspect.a005736>.

- Vanneste, E., et al. (2012). New array approaches to explore single cells genomes. *Frontiers in Genetics*, 3 (March), 44. Available from <https://doi.org/10.3389/fgene.2012.00044>.
- Vinson, B., et al. (2017). Laser direct-write based fabrication of a spatially-defined, biomimetic construct as a potential model for breast cancer cell invasion into adipose tissue. *Biofabrication*, 9(2). Available from <https://doi.org/10.1016/j.jep.2006.09.010>.
- Vogel, A., & Venugopalan, V. (2003). Mechanisms of pulsed laser ablation of biological tissues. *Chemical Reviews*, 103(2), 577–644. Available from <https://doi.org/10.1021/cr010379n>.
- Vogt, A. K., et al. (2005). Synaptic plasticity in micropatterned neuronal networks. *Biomaterials*, 26(15), 2549–2557. Available from <https://doi.org/10.1016/j.biomaterials.2004.07.031>.
- Vukicevic, S., et al. (1992). Identification of multiple active growth factors in basement membrane matrigel suggests caution in interpretation of cellular activity related to extracellular matrix components. *Experimental Cell Research*, 202(1), 1–8. Available from [https://doi.org/10.1016/0014-4827\(92\)90397-Q](https://doi.org/10.1016/0014-4827(92)90397-Q).
- Wang, W., et al. (2008). Study of impact-induced mechanical effects in cell direct writing using smooth particle hydrodynamic method. *Journal of Manufacturing Science and Engineering*, 130(2), 021012. Available from <https://doi.org/10.1115/1.2896118>.
- Wang, W., Huang, Y., & Chrisey, D. (2007). Numerical study of cell droplet and hydrogel coating impact process in cell direct writing. *Transactions of NAMRI/SME*, 35, 217–224.
- Wang, W., Li, G., & Huang, Y. (2009). Modeling of bubble expansion-induced cell mechanical profile in laser-assisted cell direct writing. *Journal of Manufacturing Science and Engineering*, 131(5), 051013. Available from <https://doi.org/10.1115/1.4000101>.
- Wang, W., Lin, Y., & Huang, Y. (2011). Modeling of thermoelastic stress wave in laser-assisted cell direct writing. *Journal of Manufacturing Science and Engineering*, 133(2), 024502. Available from <https://doi.org/10.1115/1.4003612>.
- Wilson, J. L., et al. (2014). Single-cell analysis of embryoid body heterogeneity using microfluidic trapping array. *Biomedical Microdevices*, 16(1), 79–90. Available from <https://doi.org/10.1007/s10544-013-9807-3>.
- Wood, D. K., et al. (2010). Single cell trapping and DNA damage analysis using microwell arrays. *Proceedings of the National Academy of Sciences of the United States of America*, 107(22), 10008–10013. Available from <https://doi.org/10.1073/pnas.1004056107>.
- Wu, L. M. N., et al. (2012). Increasing internodal distance in myelinated nerves accelerates nerve conduction to a flat maximum. *Current Biology*, 22(20), 1957–1961. Available from <https://doi.org/10.1016/j.cub.2012.08.025>.
- Xu, T., et al. (2006). Viability and electrophysiology of neural cell structures generated by the inkjet printing method. *Biomaterials*, 27, 3580–3588. Available from <https://doi.org/10.1016/j.biomaterials.2006.01.048>.
- Yamaguchi, S., et al. (2003). Synchronization of cellular clocks in the suprachiasmatic nucleus. *Science (New York, N.Y.)*, 302(5649), 1408–1412. Available from <https://doi.org/10.1126/science.1089287>.
- Yu, L. M. Y., Leipzig, N. D., & Shoichet, M. S. (2008). Promoting neuron adhesion and growth. *Materials Today*, 11(5), 36–43. Available from [https://doi.org/10.1016/S1369-7021\(08\)70088-9](https://doi.org/10.1016/S1369-7021(08)70088-9).
- Zhang, K., et al. (2014). Block-cell-printing for live single-cell printing. *Proceedings of the National Academy of Sciences of the United States of America*, 111(8), 2948–2953. Available from <https://doi.org/10.1073/pnas.1313661111>.
- Zheng, C. H., et al. (2012a). An integrated microfluidic device for long-term culture of isolated single mammalian cells. *Science China Chemistry*, 55(4), 502–507. Available from <https://doi.org/10.1007/s11426-012-4493-1>.
- Zheng, C. H., et al. (2012b). Quantitative study of the dynamic tumor – endothelial cell interactions through an integrated microfluidic coculture system. *Analytical Chemistry*, 84, 2088–2093.

Laser Direct-Write Bioprinting: A Powerful Tool for Engineering Cellular Microenvironments

David M. Kingsley¹, Andrew D. Dias², Cassandra L. Roberge¹ and David T. Corr¹

¹Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY, United States

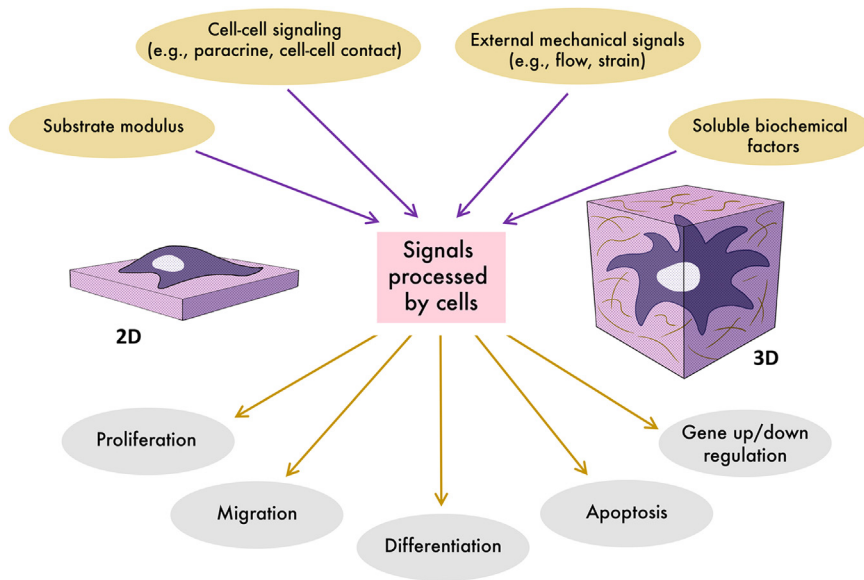
²R&D, FUJIFILM Cellular Dynamics, Inc, Madison, WI, United States

5.1 INTRODUCTION

5.1.1 SPATIAL INFLUENCES OF THE CELLULAR MICROENVIRONMENT

For tissue engineering and regenerative medicine, engineered cell microenvironments, or niches, provide a platform for directing cell fate and function. In other words, cellular behavior is influenced by the signaling of various stimuli provided by the local environment. Signals can be soluble, mechanical, and/or cellular, and the origin and transduction of each type of signal is potentially very broad and complex (Fig. 5.1). The effect and/or potency of these signaling mechanisms can be influenced by distance, time, frequency, and concentration, among other variables. This concept has been reviewed extensively (Freytes, Wan, & Vunjak-Novakovic, 2009; Godier et al., 2008; Lund, Yener, Stegemann, & Plopper, 2009) and can be employed in a variety of tissue engineering applications, such as creating cellular scaffolds/functional constructs, directing stem cell differentiation, and inducing desired cellular alignment, migration, proliferation, or protein production. There are numerous engineering approaches to produce microenvironments, such as introducing growth factors into cellular media, engineering substrates of desired material properties, creating three-dimensional (3D) hydrogels, and delivering mechanical stimuli, such as flow, tension, or compression. All of these approaches take advantage of signaling mechanisms inherent in cells, such as cellular receptors for soluble signals and transduction through the cytoskeleton for mechanical signals.

However, *in vivo* cellular communication complexity (biochemical and mechanical) is, in part, derived from the overall tissue organization, making them challenging to recapitulate within simple *in vitro* systems. The mechanisms of cell–cell signaling are spatially dependent, where cells a short distance from each other can communicate by paracrine signaling, and cells immediately adjacent can communicate by direct cell–cell contact or juxtacrine signaling. One of the challenges facing emerging applications is how to harness this complex signaling to better direct cell fate and function *in vitro*. The cellular microenvironment is further complicated by cellular population dynamics, such as the composition and distance of a neighboring population of cells, size of populations/colonies, and of course, the types of cells within the environment. The fact that neighboring cells influence cell behaviors, and that intercellular spacing is a determining factor for the mode of

**FIGURE 5.1**

Cellular signaling schematic. Cells can receive a wide variety of input signals from the microenvironment, which they process to produce any number of outputs. The combination of signals can potentially be very complex.

communication, suggest that *spatial sensitivity and controlled placement* are highly desirable in many emerging tissue engineering and regenerative medicine applications.

In addition to cell placement, control over adsorbed/encapsulated proteins may have a profound effect on cellular behavior. Cell–cell and cell–extracellular matrix (ECM) interactions are spatially sensitive, and examples of how they influence cell signaling are shown in Fig. 5.1. Whether the cellular microenvironments are planar or 3D is also very important because, depending on the application, switching from two-dimensional (2D) to 3D (or *vice versa*) can greatly impact cell signaling, function, and fate. Therefore 2D/3D approaches for engineering cellular microenvironments must provide significant spatial and compositional (e.g., density/concentration of cells, proteins, or other factors) control to guide cells' behavior. Various printing/patterning techniques may be appropriate depending on the desired application, and we will briefly examine a few, weighing their potential advantages and disadvantages. We will pay particular attention to laser direct-write (LDW).

5.1.2 OVERVIEW OF PRINTING TECHNIQUES FOR ENGINEERING CELLULAR MICROENVIRONMENTS

A number of patterning, deposition, and printing techniques have been employed to achieve spatial control in engineered microenvironments. Various biologics, including proteins, nucleic acids, and even viable cells have been successfully deposited with spatial precision. Some of the early work for

cell patterning involved controlled spatial adsorption of an adhesive protein, with subsequent seeding of cells that preferentially grow on the patterned protein. While this sort of patterning was first demonstrated using lithography-based techniques such as microcontact printing (Chen, Mrksich, Huang, Whitesides, & Ingber, 1998; Tien, Nelson, & Chen, 2002), other deposition methods like inkjet printing (reviewed in Calvert, 2001) and LDW (Colina, Serra, Fernández-Pradas, Sevilla, & Morenza, 2005; Ringeisen, et al., 2002a; Wu et al., 2003) have also shown successful protein or nucleic acid deposition. Moreover, inkjet printing (Roth et al., 2004; Saunders, Gough, & Derby, 2008; Xu, Jin, Gregory, Hickman, & Boland, 2005) and LDW (Barron, Ringeisen, Kim, Spargo, & Chrisey, 2004a; Odde & Renn, 2000; Pirlo, Dean, Knapp, & Gao, 2006; Schiele et al., 2010; Schiele, Chrisey, & Corr, 2011) have been used to deposit viable mammalian cells directly to a homogeneous substrate, without requiring the prior patterning of a protein. There are other patterning techniques available, such as dip-pen nanolithography (Piner, Zhu, Xu, Hong, & Mirkin, 1999) or atomic force microscopy-based patterning (Xie, Chung, Sow, & Wee, 2006), that focus on patterning at submicron scales. Cells certainly sense nanoscale features in their environments, but in order to study and produce cellular microenvironments where cell- and population-level interactions are controlled, patterning at the micro- and mesoscale may be most relevant for directing cellular signaling. However, patterning at this scale can be quite challenging, as these constructs are too large to be accomplished solely through chemistry, and too small to use a majority of traditional fabrication methods.

Some methods that have proven suitable for patterning at this scale include microcontact printing, LDW, and inkjet printing. Each of these has its own specific advantages and disadvantages, and they are quite complementary. Microcontact printing employs a stamp with relief features attained via photolithography. The resolution of the pattern is limited only by the wavelength of light, making submicron resolution attainable with this technique. By contrast, LDW is a noncontact technique that propels material to a substrate by laser energy absorption and partial volatilization of a sacrificial layer. The spatial location of the transferred material is determined by the programmed position of a computer-aided design/computer-aided manufacturing (CAD/CAM) stage. The resolution of this technique can be under 10 μm (Zhang et al., 2003) because of the dynamics of the material transfer event and controlled stage movement. Inkjet printing, by comparison, has a printing resolution on the order of 50 μm (Calvert, 2001), which is appreciably lower but still very good. Material deposition is achieved by one of several methods of propulsion through a nozzle (Boland, Xu, Damon, & Cui, 2006; Gonzalez-Macia, Morrin, Smyth, & Killard, 2010; Lee, Debasitis, et al., 2009; Saunders et al., 2008), and both the size of the nozzle and method of material ejection can influence printing resolution.

Because of their different mechanisms and printing resolutions, each of these methods is particularly well suited for a specific subset of applications, summarized in Table 5.1. Micropatterning excels at creating patterns of proteins on 2D flat, or even curved (Jackman, Wilbur, & Whitesides, 1995) surfaces. Cells can be seeded on adhesive proteins such that their behavior in response to the protein or protein patterns can be studied. However, once the pattern of proteins is set, it is immobilized, and cells generally do not proliferate beyond the adhesion islands. Moreover, controlled coculture is difficult because different cell types, if seeded simultaneously, will all adhere to the protein islands. Coculture patterning (reviewed in Kaji, Camci-Unal, Langer, & Khademhosseini, 2011) requires either sequential seeding on micropatterns, masking the substrate, switching regions of the substrate to be favorable to cell binding (Yamato, Kwon, Hirose, Kikuchi, & Okano, 2001; Yousaf, Houseman, & Mrksich, 2001), or combining multiple substrates (Hui & Bhatia, 2007).

Table 5.1 Patterning techniques and applications.

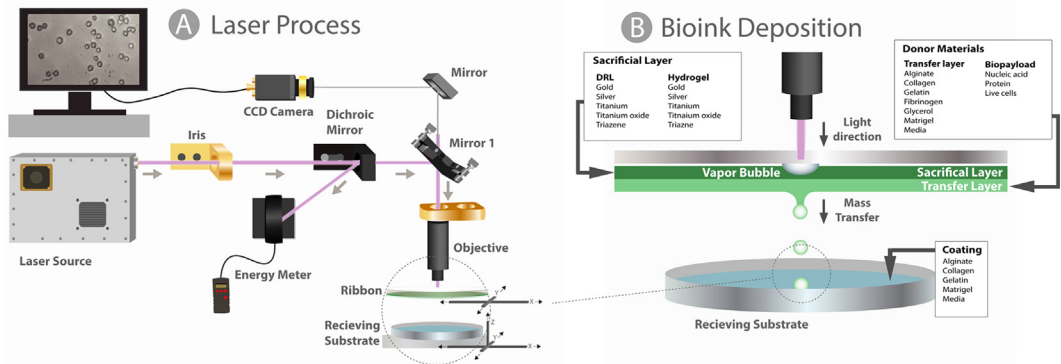
Technique	Resolution (μm)	Application
Micropatterning	<1	Patterns of adhesive proteins in 2D and cell culture on patterned adhesion islands. Study of cell behavior in controlled population or cell size and/or geometry. New mask must be fabricated for each new pattern.
Inkjet printing	~ 50	High-throughput or large constructs in 2D or 3D on any suitable substrate. Direct patterning of cells or less viscous materials.
Laser direct-write	~ 10	Patterning of cells or material in 2D or 3D on any suitable flat substrate. Direct patterning of cells with high spatial resolution. More viscous materials can be patterned, but with lower throughput than inkjet printing.

On the other hand, inkjet printing and LDW do not require patterning of adhesive proteins to control cellular placement. Therefore cells can be directly deposited to a homogeneous substrate, allowing evolution of a printed structure from a prescribed initial condition. Furthermore, both inkjet printing and LDW have also been extended to print in 3D, allowing 3D spatial control over the microenvironment. In inkjet printing, the biologic payload (e.g., cells, proteins, or other biomolecules) is printed through the controlled deposition of solution containing the desired payload, much like the multiple colors of ink in color printing. In this way, the amount of payload-containing solution, and its placement, can be precisely controlled to rapidly generate patterns with multiple cell types or materials. However, the location of the cells, or other biopayload, within the areas of dispensed solution is not controlled. For discrete components like cells, the payload is randomly distributed within a liquid volume, although parameters such as concentration are controllable. Therefore inkjet printing is particularly well suited for rapidly fabricating larger patterns, including constructs of intricate geometries and multiple cell types, in which the geometric precision is important, but spatial control of the cells or groups of cells is not.

In a traditional LDW setup, a camera is coincidentally focused with the laser, allowing direct visualization of the biologic payload on the print ribbon. As a result, the specific cell or group of cells (or other biopayload) to be printed can be visualized, targeted, and transferred to the substrate with high spatial precision. In contrast to inkjet printing, where a controlled volume of liquid is deposited from a reservoir, LDW allows targeting of specific print regions on the ribbon, so the dispersal of cells within the volume transferred is *not* random. LDW is also capable of printing more viscous materials that may not be dispensed by an inkjet nozzle. This unique ability to combine the high-resolution placement of selected biological payloads with various substrate materials makes LDW particularly attractive to engineer *in vitro* cellular microenvironments.

5.1.3 LASER DIRECT-WRITE OVERVIEW

LDW is a noncontacting material deposition technique. Various methods for laser-based deposition have been utilized, each quite similar, but with some distinct (although often subtle) differences. In this chapter, we will group all of these methods under “LDW,” although there are different preferences in the field about the most appropriate term to use. LDW-based methods operate on the same general principle, illustrated in Fig. 5.2. A typical LDW setup consists of two coplanar plates: a

**FIGURE 5.2**

(A) Schematic of typical LDW setup for material or cell deposition. A laser beam passes through beam-delivery optics and a laser-transparent print ribbon to interact with material, partially volatilizing a sacrificial layer and forming a vapor pocket to eject the transfer material and biopayload to a receiving substrate. Independent CAD/CAM control of the ribbon and receiving stages allows programmatic deposition of material. (B) Materials utilized in LDW. The laser first interacts with a thin sacrificial layer, which can be either a hydrogel or a dynamic release layer (DRL) such as triazene, metals, or metal oxides. The biologic payload is typically suspended in a transfer layer (e.g., glycerol for printing nucleic acids or proteins; hydrogel or media for printing live cells). For cellular printing, live cells can either be attached to the material to be transferred (e.g., when using Matrigel), or suspended in hydrogel or media in their trypsinized state. A thin coating is also typically utilized on the receiving substrate to provide viscous energy dissipation.

Adapted from Kingsley, Roberge, et al. (2019), with permission.

laser-transparent print ribbon, which holds the material/cells to be deposited, and a receiving substrate onto which material is printed (Fig. 5.2A). The major difference among the various LDW methods is their choice of laser source. Indeed, a variety of lasers have been utilized for bioprinting ranging from 193 to 1064 nm with pulse durations typically on the femto or nanosecond range. Both the receiving substrate and print ribbon can be independently moved with CAD–CAM-controlled stages. A charge-coupled device camera also allows real-time visualization of the ribbon and receiving substrate. The underlying side of the mounted ribbon is coated with two thin layers, the first is a sacrificial, energy-absorbing layer, and the second a transfer layer. The sacrificial layer directly interacts with the laser, while the transfer layer consists of the material to be printed. Energy-absorbing layers are selected based on the laser wavelength to enhance the release of print material. A pulse from the laser passes through the transparent ribbon and volatilizes the sacrificial layer, ejecting the transfer material onto the substrate below (Fig. 5.2B).

The consensus mechanism for deposition is that laser energy is absorbed by the sacrificial layer, forming a vapor pocket at the ribbon–material interface (Barron, et al., 2004a). Expansion of the vapor pocket allows the printed material—the donor material—to form a droplet that is ejected from the surface of the print ribbon, on a trajectory perpendicular to the plane of the ribbon. It is also generally accepted that for a high-power laser and an appropriate sacrificial layer, mass transfer occurs at a much faster time scale than heat transfer (Barron, et al., 2004a). Furthermore, heat

shock protein expression does not appear to be elevated with LDW (Chen, Barron, & Ringeisen, 2006), meaning that thermal damage to the transferred material is negligible. Moreover, the laser–material interaction occurs at the surface of the material (Barron, et al., 2004a; Barron, Wu, Ladouceur, & Ringeisen, 2004b), so the bulk of the material that is transferred using LDW never directly interacts with the laser. After deposition, the receiving substrate is moved to the next programmed position, and a new spot is used for volatilization on the ribbon. Serial deposition creates a desired structure or pattern according to the programmed stage positions. Although the receiving substrate is typically a Petri dish, cover slip, or glass slide, which is often coated with a thin layer of material that serves as the matrix material, as a technique LDW is independent of the print surface, making it compatible with a wide range of receiving substrates.

5.2 MATERIALS IN LASER DIRECT-WRITE

5.2.1 MATERIAL PROPERTIES INFLUENCING CELLULAR MICROENVIRONMENTS

In this section, we will focus on how the materials used in LDW can affect cell response and thus be used to engineer desired microenvironments. Functional and mechanical properties of the material will determine not only how the cells interact with the substrate but also how they interact with other nearby cells.

Mechanical properties of a substrate play a critical role in the gene expression and determination of the functional role a cell performs. As cells adhere to a substrate, they form focal complexes that become points of force transfer between the substrate and the cell. On a softer substrate, in response to a stress initiated either externally or by the cell itself, the substrate–cell attachment region deforms more than the cell. On stiffer surfaces, less deformation of the substrate interacting with the focal contact point takes place, and there is a greater stress on the cytoskeleton elements causing cell changes. Significant differences in substrate stiffness manifest in different cell morphologies, cytoskeletal reorganization, gene expression, and fate decisions. The pioneering work of Dennis Discher’s group (Engler, Sen, Sweeney, & Discher, 2006) showed that mesenchymal stromal cells, when cultured on substrates of different elastic moduli, exhibited different morphologies and gene profiles; substrate stiffness could be used to direct their differentiation (Engler et al., 2006). In more recent work, it has been further shown that substrate stress relaxation behaviors may provide equally important stimuli to cells (Chaudhuri et al., 2015). Thus, the substrate mechanical properties are an important factor to consider when engineering the cellular microenvironment.

Functional groups present and their relative concentration in the ECM also play a pivotal role in the overall behavior of the cell. The presentation and binding of ligands is necessary to induce adhesion, motility, survival, differentiation, and other specific cell functions. For example, human embryonic stem cell (hESC) culture was developed using a Matrigel substrate, a hydrogel derived from the mouse tumor basement membrane. However, synthetic substrates can be functionalized with the integrin ligands found within Matrigel to support hESC attachment and growth (Liu, Wang, Kaufman, & Shen, 2011). In addition to the presence of the specific functional groups, their relative concentrations also play a key role in cell behavior. Substrates modified with different surface concentrations of the arginine–glycine–aspartic acid (RGD) motif exhibit differing degrees of

cellular attachment, affecting the cytoskeletal structure, its organization, and the cellular morphology (Massia & Hubbell, 1991). Another feature of engineered motifs is that binding affinity is different from that of the same motif within a natural protein, such as collagen or fibronectin. As a result, engineered substrates may have different binding kinetics than natural substrates. However, the surface-coated RGD can be engineered to contain additional peptide sequences that enhance its specificity or binding kinetics to a ligand (Petrie, Capadona, Reyes, & García, 2006). Overall, mechanical properties of the material, such as elastic modulus, and biochemical properties of the material, such as functionalization, can have a profound influence on cells within the microenvironment.

5.2.2 MATRIGEL-BASED LASER DIRECT-WRITE

Initial studies of cell printing by LDW utilized Matrigel as a coating on the receiving substrate, as well as on the ribbon as a sacrificial layer/transfer material (Fig. 5.2B). Matrigel is a soluble tumor extract composed of an assortment of proteins, and it undergoes thermal gelation at 37°C. (Kleinman et al., 1986). There are several features that make Matrigel an attractive candidate material for both the receiving substrate and as a transfer material for LDW studies. Matrigel contains large quantities of essential structural proteins along with many of the other proteins, proteases, and growth factors found in the basement membrane (Kleinman & Martin, 2005). Matrigel has been used widely for *in vitro* cell culture to differentiate a variety of cells, as well as for hESC culture (Xu et al., 2001). Additionally, Matrigel provides a permanent matrix to immobilize printed material on the receiving substrate, contributing to the high pattern fidelity.

However, despite these many benefits, Matrigel has a number of shortcomings that may preclude or limit its use in certain applications, causing some researchers to seek alternative ribbon and substrate materials. As a transfer material, Matrigel requires a cell-based biopayload to loosely attach to the Matrigel matrix. Cellular attachment times vary depending on the cell type, making co-culture transfers difficult and limited to adherent cell types. As an ECM-mimetic substrate material, Matrigel has an inherent batch-to-batch variability because it is grown and extracted from a mouse tumor. This variability may be a source of inconsistencies among researchers, and can make it difficult to reproduce previous findings. Further, not all Matrigel components are defined, and some concentrations are unknown. Without knowing Matrigel's exact content and concentration of proteases, growth factors, and other proteins, the amount of information that may be inferred from an experiment is largely limited. In an experiment using Matrigel, it is impossible to know what factor or factor combination is responsible for the observed cellular behavior, since any potential influence by Matrigel's numerous unspecified constituents cannot be ruled out. Although Matrigel provides a cost-effective basement membrane substitute because it is derived from mouse sarcoma cells, it could elicit an extreme immune response if implanted into the body. Moreover, extrapolating cell behavior observed on a mouse tumor extract to a human condition could be inaccurate.

Several synthetic Matrigel alternatives have emerged, and are continuing to be developed within the biomaterials field, that can immobilize cells, which is critical to enabling printed pattern fidelity. Some alternatives include polyacrylamide and poly(ethylene glycol) (PEG) (reviewed in Aisenbrey & Murphy, 2020). A major advantage of synthetic materials is their independently tunable material properties, such as elastic modulus, permeability, degradation kinetics, and peptide functionalization (Anseth & Lin, 2009; Toepke, Impellitteri, Theisen, & Murphy, 2012). However,

printing applications impose additional constraints to the biomaterials used, notably the material viscosity with nozzle-based extrusion methods, and gelation with all printing methods. The challenge is that a high-viscosity material may be needed to achieve the desired printed material stiffness, yet it can be quite difficult to print high-viscosity materials. For this reason, gelation after printing is typically preferred. However, many of the tunable characteristics of synthetic hydrogels are achieved with covalent hydrogel crosslinking, which is slower than ionic or electrostatic gelation, and thus may influence print fidelity. Gelation kinetics are important to consider when choosing a material for printing.

One of the benefits of Matrigel, along with other commonly used bioinks, is consistent and relatively quick gelation. On the other hand, with PEG, chemical-based gelation can take hours to occur (Tan, DeFail, Rubin, Chu, & Marra, 2010), yet this can be greatly accelerated to the scale of minutes by enabling photo-crosslinking hydrogels through the use of click chemistry. While not yet demonstrated with LDW, PEG has found utility in culturing mesenchymal stromal cells in extruded gels (Xin, Chimene, Garza, Gaharwar, & Alge, 2019). Blends of PEG with other biomaterials, that is, PEG blends, permit harnessing the tailorable mechanical properties of PEG with functional and gelation properties of other materials. Some of these copolymers and composites used in bioprinting applications include a PEG-Fibrinogen copolymer for generating myotubes (Constantini et al., 2017) and a PEGDA/nanoclay/hyaluronic acid composite to promote osteogenic differentiation (Zhai et al., 2018). These advances highlight the tunability and compositional control offered by engineered materials, hence incorporating such Matrigel alternatives is a logical next step for LDW bioprinting.

5.2.3 GELATIN-BASED LASER DIRECT-WRITE

Among the hydrogel materials explored for use with LDW, gelatin has proven quite promising. Gelatin is a natural polymer that is a reduced and degraded form of collagen. At room temperature, gelatin is a stiff gel, but at incubation temperatures (37°C) the polymer network becomes soluble. In LDW applications, gelatin is currently used both as a sacrificial and transfer material, as well as a receiving substrate (Dias, Unser, Xie, Chrisey, & Corr, 2014; Schiele et al., 2011). As previously described, a laser pulse vaporizes the sacrificial gelatin, ejecting a droplet of cells. What is truly powerful about this approach is its exploitation of the thermally reversible properties of gelatin. On the print ribbon, exploiting gelatin's thermal properties allows for the partial encapsulation of the biopayload, giving the opportunity for a wide variety of cells and other payload to be transferred. Further, this technique does not require cells to attach to the matrix on the ribbon (as with Matrigel), and thus allows cells to be printed in their suspended state. Printing suspended cells does not disrupt focal adhesions, and therefore minimizes cell trauma compared to printing attached cells. In addition to gelatin, it is possible to use other materials with temperature-dependent gelation or viscosity, such as glycerol (Guillemot et al., 2010), to encapsulate the biopayload for transfer. Gelatin's thermal properties also offer unique benefits when used on the receiving substrate. Following transfer, after a brief incubation period, the gelatin on the receiving substrate is liquefied, leaving only the LDW-patterned cells. This further takes the advantage of gelatin's thermal reversibility to minimize the influence of potentially unwanted matrix factors in simple cell studies. The versatility of gelatin can be further enhanced through methacrylation, to yield gelatin methacryloyl (GelMA). The methacryloyl functionalization enables gelatin to be rapidly photocrosslinked in the

presence of a photoinitiator (Loessner et al., 2016; Yue et al., 2015). The result is a 3D structure that keeps its RGD peptide sequence that favors cell behaviors (adhesion, proliferation, and differentiation) and may be degraded by cell-produced matrix metalloproteinases. These features and its tunable mechanical properties have made GelMA an ideal bioink within many bioprinting applications (Liu et al., 2017; Ouyang et al., 2020); however, GelMA has not yet been utilized in LDW.

5.2.4 DYNAMIC RELEASE LAYERS

Sacrificial material on the print ribbon enables a volatilization during an LDW deposition event without sacrificing biologics or cells. An appropriate energy-absorbing material is selected based on the absorption coefficient at the applied wavelength. High-wavelength near-infrared laser sources (e.g., 1064 nm) typically use a metal or foil as a sacrificial layer, whereas UV lasers often employ a biologic matrix, such as Matrigel or gelatin (Koch, Brandt, Deiwick, & Chichkov, 2017). Both methods have shown success depositing multiple mammalian cell types in controlled patterns (Table 5.2). A dynamic release layer (DRL) on the ribbon is often used to control the material interacting directly with the laser, decoupling the laser from the transfer material. DRLs are thin, sacrificial layers of material that interact with the laser at its operational wavelength, and can amplify the energy from the laser (Fig. 5.2B). Some example materials for this layer are triazene or a metal/metal oxide (Ringeisen et al., 2008; Schiele et al., 2009). With LDW, the size of the transferred material droplet is directly related to laser fluence. However, inconsistencies of the transfer material on the ribbon, such as uneven coating, could produce unwanted spot-to-spot variation in the pattern. The use of a DRL has been shown to help minimize the energy threshold required for transfer and decrease the thermal impact on the transfer material (Banks et al., 2008). The other benefit of using such a DRL is that it provides a consistent material at the ribbon interface, thereby granting a more predictable laser–material interaction when utilizing a variety of transfer materials. Without a DRL, each transferred material will exhibit different transfer dynamics due to specific laser–material interactions. The use of a DRL will result in a consistent vapor pocket, independent of the transfer material. However, utilizing a DRL may impair or preclude ribbon and substrate visualization.

5.2.5 ADDITIONAL HYDROGELS USED FOR PRINTING AND THE RECEIVING SUBSTRATE

An extensive number of hydrogels and hydrogel blends have been used for LDW applications (Fig. 5.2B). The use of a DRL has made it simple to change between different hydrogel materials on the ribbon as a transfer material for cells or other biological contents. To date, the most commonly used hydrogels as a transfer material are gelatin, Matrigel, and alginate (Barron, et al., 2004a; Koch et al., 2010, 2012). The same hydrogels are also commonly used to coat the receiving substrate during printing, to cushion the impact of printed cells, and to immobilize the printed spots to increase pattern fidelity. The feature that these hydrogels have in common is that they have rapid and controllable gelation properties that benefit immobilization of the biopayload. Fibrinogen/hyaluronic acid and collagen have also been used both as transfer materials and for coating the receiving substrate (Grune, et al., 2011a; Koch et al., 2012). Current research in LDW has not yet

Table 5.2 Examples of cell types and materials successfully deposited by LDW, illustrating the applicability and broad range of research.

Sacrificial layer	Transfer layer	Substrate	Cell type	References
Matrigel	Matrigel/media	Matrigel	Human osteoblast; Chinese hamster ovary (CHO) cells; mouse embryonal carcinoma (P19); bovine pulmonary artery endothelial cells, rat neural stem cells, mouse myoblast	Ringeisen, et al. (2002b) , Ringeisen et al. (2004) , Schiele et al. (2009)
Gelatin or alginate/gelatin	Gelatin/media, cell-encapsulating microbeads	Gelatin, mesentery tissue	Human dermal fibroblasts, mouse embryonic stem cells (mESC), human breast cancer cells, adipocytes	Kattamis, Purnick, Weiss, and Arnold (2007) , Schiele et al. (2009, 2011) , Raof, Schiele, Xie, Chrisey, and Corr (2011) , Dias et al. (2014) , Phamduy et al. (2015) , Vinson et al. (2017)
	Alginate	Gelatin / calcium chloride	Human breast cancer, mESC, NIH 3T3 mouse fibroblast	Kingsley, Dias, Chrisey, and Corr (2013) , Kingsley et al. (2016) , Xiong et al. (2017) , Kingsley et al. (2019)
Metal: Au, Ag, Ti, TiO ₂	Matrgel, alginate, glycerol, fibrinogen, collagen, laminin, hyaluronic acid, media	Gelatin, matrigel, media, PEG scaffold, thrombin-fibrinogen, matriderm, <i>in situ</i> printing	Rat (schwann, astroglial); pig lens epithelial, human osteosarcoma (MG 63); rat cardiac; mouse endothelial, ovine endothelial cells, ovine vascular smooth muscle cells, 3T3 fibroblasts, human keratinocytes; human adipose-derived stem cells, endothelial colony forming cells (ECFC), multipotent mouse bone marrow stromal precursor cells, human embryonic stem cells	Hopp et al. (2005) , Barron et al. (2004a) , Barron et al. (2004b) , Barron, Krizman, and Ringeisen (2005) , Doraiswamy et al. (2006a) , Chen et al. (2006) , Ovsianikov et al. (2010) , Koch et al. (2010) , Wu and Ringeisen (2010) , Gaebel et al. (2011) , Gruene, et al. (2011b) , Guillemot et al. (2010) , Koch et al. (2012) , Keriquel et al. (2017) , Sorkio et al. (2018) ; Gruene et al. (2011c)
Triazene	Matrigel	Matrigel	Rat B35 neuronal neuroblast cells	Patz et al. (2005) ; Doraiswamy et al. (2006b)

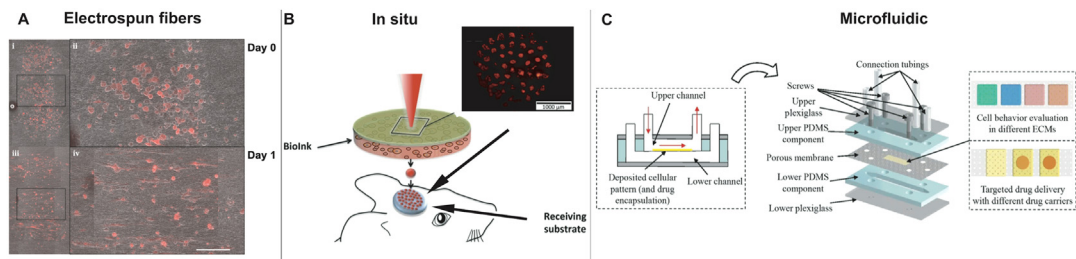
focused on customizing receiving substrate properties, which can be achieved using a combination of natural and synthetic hydrogels.

Hydrogels as substrate coating materials allow for the manipulation of both the mechanical and biochemical properties of the environment. The biochemical properties of the surface can be engineered either by using protein-based gels or grafting functional moieties into the polymer that makes up the gel. Additionally, the mechanical properties of the hydrogel are tunable by manipulating the amount of crosslinking and the relative polymer concentration (Kingsley, McCleery, et al., 2019; Kingsley, Roberge, et al., 2019; Tse & Engler, 2010). LDW applications have not yet explored this parameter space. However, nonpatterning applications have begun customizing both of these properties in hydrogels for a wide range of applications. One example of manipulation of hydrogel biochemical and mechanical properties is within alginate gels, where the elastic modulus and RGD grafting density were independently manipulated to optimize the environment for stem cell differentiation (Huebsch et al., 2010). Printed material can also be manipulated through *in situ* crosslinking on the receiving substrate. Example of this include the printing of cells suspended in fibrinogen to substrates of thrombin, which after a period of incubation forms fibrin (Grune, et al., 2011a), and the printing of alginate into a substrate of calcium to fabricate and localize microbeads (Kingsley et al., 2013).

5.2.6 NONHYDROGEL RECEIVING SUBSTRATES AND SYNERGISTIC TECHNOLOGIES

Another means to control the receiving substrate surface properties is through the use of engineered nonhydrogel materials or scaffolds. One such example of this is electrospun nanofibrous structures. As a surface coating material, electrospun fibers can be fabricated with a variety of natural and synthetic polymers, where fabrication parameters allow for the tuning of stiffness, topography, degradation, and fiber size (Pham, Sharma, & Mikos, 2006). Fibers can also be chemically treated after fabrication with desired functional units, similar to protein grafting in hydrogels. Additionally, electrospun fibers can be fabricated in such a way that they are aligned, giving cells directional cues (Schaub, Britton, Rajachar, & Gilbert, 2013). Utilizing electrospun fiber substrates with cell printing is another way to achieve idealized microenvironments, with the addition of directing cell growth through properties of the fibers and their structural alignment. A 1-day time course of fibroblasts printed on electrospun fibers (Fig. 5.3A) indicates that cells maintain registry to the printed pattern. Aligned fibers appear to direct the cell elongation and migration after LDW. Printing onto substrates with topographical features enables the fabrication of unique constructs or cell studies that are difficult to perform using other techniques.

Living substrates have also been investigated in conjunction with laser-based bioprinting. One potential strategy is to directly harvest tissue and utilize it as a substrate *ex vivo*. In work from the Chrisey lab, harvested tissue slices served as LDW printing substrates for an *in vitro* platform to observe cancer cell invasion in the presence of an angiogenic substrate (Phamduy et al., 2015). Another area of investigation involves printing directly into a living organism, for example, wound healing or tissue reconstruction. The potential for this type *in situ* laser-based bioprinting has been demonstrated by Guillemot and colleagues printing into mice to repair cranial defects (Fig. 5.3B) (Keriquel et al., 2017). Taken together, there are numerous interesting possibilities for living substrates, *in vivo* and *ex vivo*.

**FIGURE 5.3**

Nonhydrogel receiving substrates for LDW. (A) RFP human dermal fibroblasts printed by LDW onto PLLA electrospun nanofiber substrates. Nanofibers are aligned on the substrate, oriented left-to-right in images. Fibroblasts (i and ii) immediately after printing appear rounded in their suspended state, but (iii and iv) after one day begin to align in the direction of the electrospun fibers. Scale bars are 200 μm . (B) LDW printing *in situ* for the repair of a cranial defect and to promote healing. (C) LDW performed within microfluidic devices for use as an *in vitro* diagnostic tool.

(B) Adapted from Keriquel et al. (2017), with permission; (C) Adapted from Xiong, Chai, and Huang (2016), with permission.

Another strategy to control the microenvironment utilizes microfluidic devices as printing substrates. Microfluidic devices, for example, lab-on-a-chip and organ-on-a-chip, have been used for models of tissues, disease, organs, and multi-organ systems (Agarwal, Goss, Cho, McCain, & Parker, 2013; Bavli et al., 2016; Jang et al., 2013; Kim, Huh, Hamilton, & Ingber, 2012; Oleaga et al., 2016). These devices are favored because of their powerful ability to mimic key tissue functions with flow and mechanical forces, for example, blood–brain barrier and lung (Huh et al., 2010; van der Helm, van der Meer, Eijkel, van den Berg, & Segerink, 2016). The combination of bioprinting technologies with organ-on-a-chip offers powerful potential. Huang and colleagues have shown these substrates are compatible with laser-based bioprinting technologies for rapid prototyping and screening functions (Fig. 5.3C) (Xiong et al., 2016). These microfluidic platforms have also been integrated with inkjet-based bioprinting to produce a multi-organ-on-chip model. In one such study, bioprinting in microfluidic devices was used to produce several tissue surrogates that shared a circulatory system (Skardal et al., 2017). The linking of these devices enabled researchers to observe the effects of drug screening on a target tissue and the downstream effects on other secondary organs.

5.3 LASER DIRECT-WRITE APPLICATIONS IN 2D

LDW was first used in 2D for electronics applications (Chrissey et al., 2000). Once it was shown that LDW could be adapted for use in soft materials transfer and deposition of biologics, 2D printing of nucleic acids (Colina et al., 2005; Fernández-Pradas, Colina, Serra, Dominguez, & Morenza, 2004), proteins (Dinca et al., 2008), and even live cells (Wu et al., 2001) was demonstrated. Moreover, custom configurations of cells, such as grids, lines, and sheets have been demonstrated, with unrestricted cell growth from the initial printed pattern (Fig. 5.4). These initial studies showed

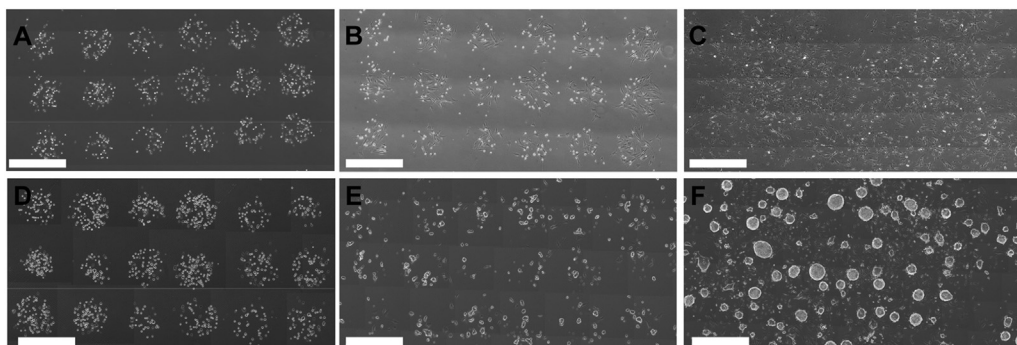


FIGURE 5.4

Examples of cells printed by LDW, and evolution of structure over time. Array of human dermal fibroblasts (A) immediately after printing, (B) 1 day after printing, and (C) 3 days after printing showing the evolution of cellular network structure from the initial printing positions. Array of mouse embryonic stem cells (D) immediately after printing and (E) 1 day after printing, and (F) 7 days after printing, demonstrating the formation of embryoid bodies (EBs) on an unrestricted, uniform substrate due to collective cellular aggregation, rather than constrained growth. Scale bars are 500 μm .

the many promising applications, ranging from biosensor fabrication, to the creation of small grafts or biological constructs, to building spatially precise cultures for *in vitro* diagnostics and cellular signaling studies that we will discuss herein.

Because the substrate is generally homogeneous, following LDW, cells are free to migrate, cluster, or form structures uninhibited by geometric or biochemical restrictions on the substrate. This property of LDW allows cellular migration and migration-based behavior to be studied. By observing structural evolution, LDW enables different types of studies than what can be explored using micropatterned proteins, because patterning proteins restricts cell migration to adhesion islands. Unrestricted cell growth following LDW has been demonstrated with a Matrigel coating on the substrate in earlier work (Schiele et al., 2009; Wu et al., 2003), and more recently with a gelatin substrate coating (Schiele et al., 2011). An advantage of the gelatin substrate is that the gelatin coating is temporary (as mentioned in Section 5.2.3), and does not provide cells with a permanent scaffold or a large assortment of unknown complex signaling factors. Despite the temporary nature of the gelatin layer, cells printed to the substrate maintain excellent pattern registry, moving on average, less than 6 μm from their initial location within half an hour of printing (Schiele et al., 2011). One restriction of this technique is the limitation to a flat surface parallel to the ribbon, but otherwise potentially any cell-adhesive material can be used with LDW, such as hydrogels or scaffold materials with engineered topography.

Although LDW can potentially be used to study the effects of cell location and cell–cell interaction, exploration of cellular phenomena after LDW patterning has been examined in only a few instances. One study of ESC behavior examined the clustering of ESCs after LDW. ESCs are pluripotent, meaning they can differentiate into any of the three primitive germ layers. It was shown that following LDW, mESCs maintained their pluripotency, as evidenced by their ability to express markers of all three primitive germ layers (Raof et al., 2011). An additional indication of

pluripotency is the formation of 3D cell clusters called EBs, which contain cells of all three germ layers. The cell–cell interactions within the EB are of great importance. The size of the EB, which may impart complex signals to cells within the structure, can influence differentiation, as can the size of a stem cell colony (Bauwens et al., 2008; Lee, Peerani, et al., 2009; Peerani et al., 2007). The micropatterning approach previously used to study this phenomenon only allows the combined effect of the patterned protein and colony size to be studied. However, it would be ideal to study the effect of colony size and the surface-coated protein independently.

Controlling EB size can potentially be very useful to direct differentiation, and LDW has been used to control EB size, via the density of printed cells, independent of the stem cell colony diameter (Dias et al., 2014). While colony size did not influence the size of the EBs that formed, the effect of colony size on stem cell differentiation based on cellular patterning on a homogeneous substrate still needs to be determined. Prescribing these factors in engineered microenvironments could allow more efficient directed differentiation of stem cells. Additionally, differentiation can also be influenced by printing protein gradients, as reviewed (Tasoglu & Demirci, 2013). The versatility afforded by LDW for printing cells, biomaterials, and proteins, enables complex studies to differentiate stem cells, influence migration, and answer many other questions using engineered microenvironments.

5.4 LASER DIRECT-WRITE APPLICATIONS IN 3D

5.4.1 MICROENVIRONMENTS IN 3D

Planar, 2D cell culture has long been a paradigm for studying mammalian biological phenomena *in vitro*, ranging from stem cell differentiation and tissue development to drug testing. However, fundamental differences exist in the way cells behave between 2D and 3D microenvironments. Cells with 3D microenvironment interactions show differences in their cytoskeletal structure, morphology, membrane protein distribution, and interaction with soluble factors (Pampaloni, Reynaud, & Stelzer, 2007). Additionally, cells cultured within a 3D ECM experience limitations on cell migration, while those grown on a 2D substrate can migrate and proliferate without the same restrictions.

In a 2D environment, cells are only able to interact and attach to the ECM on the substrate. This produces a difference in the receptor density and orientation along the surface of the cell compared with receptor orientation in 3D (Meshel, Wei, Adelstein, & Sheetz, 2005). Further, the composition and strength of the complexes forming the adhesions differ between 2D and 3D microenvironments. As an example, on a 2D substrate, fibroblasts form adhesions along only the ventral surface (Berrier & Yamada, 2007). The localization of cell traction forces results in a morphological polarity that does not exist in 3D. In a 3D environment, adhesion is formed via focal complexes (as opposed to 2D focal adhesions), all along the cell membrane, and has a different composition. The difference in cell distribution and type of adhesion site in 3D affects the organization and generation of tension in the cytoskeleton (Pedersen & Swartz, 2005). The compounding discrepancies between 2D and 3D cell–matrix interactions can yield very different behaviors and response to stimuli.

In a 3D ECM, cell migration occurs either by moving through the material's pores, or by breaking down the surrounding ECM with proteases. Highly porous materials, such as sponges and foams, typically have pore sizes greater than the cell diameter, allowing for nonproteolytic migration. In hydrogels, the pores are on the nanoscale (much smaller than the actual cell). For infiltration to occur, the cell must produce proteases to break up the restrictive matrix, assuming the matrix is made from a peptide-based gel. The ease of infiltration will determine the migratory rate of the cells within the material. Additionally, the pore size will be a factor in determining the rate that nutrients can diffuse into and waste products can flow out of the bulk material. If the bulk material is too thick and/or pore size too small, toxins can build up in the environment, or cells can die due to ischemic effects.

Fundamental biological questions have been, and will continue to be, solved using 2D culture models. However, 3D alternatives are necessary to overcome 2D matrix interactions that fundamentally change cell morphology and behavior (e.g., cytoskeletal structure, membrane protein distribution, and interaction with soluble factors, and focal adhesions) (Pampaloni et al., 2007). Additionally, the creation of large tissue-engineered constructs, for fundamental research or *in vivo* transplantation, will require a means of 3D fabrication with the precise spatial arrangement of the contents. To overcome the limitations set by 2D environments, LDW has been adapted to build more physiologically relevant 3D culture models, *in vitro* diagnostic tools, and tissue-engineered constructs.

5.4.2 LAYER-BY-LAYER APPROACHES

Layer-by-layer (LbL) printing is a 3D biofabrication approach adapted from industrial rapid prototyping technologies. Traditionally, LbL printing utilizes a 2D method, sequentially, to produce an overall 3D construct. After one layer is fabricated, it is often stabilized before the next layer is printed to provide a flat new printing surface. For LbL LDW, liquid hydrogel, or hydrogel precursor suspending a desired biologic, is used as a transfer material, similar to the 2D printing method. The hydrogel is deposited at preprogrammed coordinates, according to the specific layer design. Once the printed layer is completed, it is gelled by either crosslinking the polymer solution, or inducing polymerization. Gelation can be triggered by a number of different crosslinking or polymerization mechanisms (e.g., thermal, ionic, pH, or enzymatic), depending on the hydrogel or hydrogel blend. The rate of gelation for each of these mechanisms differs, and generally a fast gelation time is desired to maintain pattern fidelity. Once the layer is gelled, the printing surface is stabilized, and the process can be repeated to add additional layers until the overall construct is completed.

Another method of performing LbL fabrication with LDW utilizes a hydrogel precoated on the substrate, rather than in the transferred material. Thin-film coating mechanisms can consistently make thin layers of liquid hydrogel polymer or precursor on a substrate at a desired height. The selected material is transferred into the liquid hydrogel layer in a programmed pattern. The layer is gelled, and a new printing layer is produced by the addition of new hydrogel solution, again coated to the desired layer thickness. This technique is very similar to the previous method, but may hold advantages if each layer needs only a small amount of printed substance, relative to the overall bulk material. These techniques can be repeated for a desired number of layers. 3D resolution is determined by the hydrogel coating mechanism's control over the height of the newly laid hydrogel

layer. The average height for individual layers with one such coating technique, blade-coating, approaches approximately 40 μm (Gruene, et al., 2011b). Combinations of hydrogel materials previously used in LbL LDW, as well as other candidate materials, have been listed in Fig. 5.2. LbL LDW has been used for *in vitro* and *in vivo* skin tissue, an osteosarcoma model, cardiac regeneration, and corneal tissue (Catros et al., 2011; Gaebel et al., 2011; Koch et al., 2012; Sorkio et al., 2018). Beyond creating tissue models, 3D LDW can be used to study cell-to-cell signaling. One powerful example of 3D LDW studied the coculture signaling and migration between adipose-derived stem cells and ECFCs in hyaluronic acid and fibrin gels (Gruene, et al., 2011a).

The LbL LDW printing technique appears to be very similar to another LbL technique, inkjet printing, which has also been adapted for biological applications. Inkjet printing deposits hydrogel material, which suspends cells, directly from a nozzle to a substrate, either spot by spot or by continuous flow. Discrete layers of printed material are often gelled prior to printing additional layers. The advantages of inkjet printing include that it is generally less expensive than LDW and offers higher throughput.

5.4.3 LASER DIRECT-WRITE MICROBEADS

Microbeads are spheroidal microstructures that can encapsulate a desired biologic, and have been investigated for applications ranging from drug delivery to cell culture (Amsden & Goosen, 1997; Xie & Castracane, 2009). For cell-based applications, microbeads are fabricated from cross-linkable polymers, or precursor as used for hydrogels. Like bulk hydrogels, microbeads provide cells with a 3D environment, but there are distinct advantages to the microbead structure. Nutrient diffusion into bulk hydrogels occurs slowly, and large gels may even require a bioreactor to prevent encapsulated cells from suffering from ischemic effects or buildup of metabolic waste. Microbeads, on the other hand, have a favorable surface area-to-volume ratio, allowing for rapid exchange of nutrients and waste products. Further, microbeads provide greater control of the cell microenvironment, especially in studies involving multiple cell types. Properties of the individual beads can be tailored for the specific cell type, whereas bulk gels are generally isotropic.

Current microbead fabrication techniques include electrostatic bead generators, microfluidic devices, and other emulsion-based techniques (Amsden & Goosen, 1997; Desmarais, Haagsman, & Barron, 2012). These technologies operate under the same general principles to fabricate beads: cells are suspended in a polymer solution, and a single droplet is extruded into a crosslinking bath, where rapid gelation occurs to encapsulate the suspended contents. For example, in a solution of alginate with suspended cells, a droplet can be extruded into a bath containing calcium. The divalent Ca^{2+} cation ionically cross-links the alginate, causing rapid gelation, and encapsulating the suspended cells. The current fabrication technologies used to produce microbeads have certain limitations in their uses. Electrostatic bead generators limit bead fabrication to only polyelectrolyte materials, whereas other pressure- and flow-based techniques appear to have relatively consistent control of bead size, but only over a limited range. None of these technologies are able to control microbead placement.

A recently developed method for bead fabrication uses LDW to both fabricate and pattern cell-containing alginate microbeads in a single step (Kingsley et al., 2013). This LDW setup consists of a ribbon with an alginate–gelatin sacrificial layer and cell-suspending alginate transfer material. The receiving substrate consists of a thinly coated layer of gelatin/calcium chloride. A pulse from a

laser ejects cell-containing alginate droplets to the substrate below in a prescribed location, where *in situ* gelation occurs via calcium chloride/alginate crosslinking. This technique manipulates microbead size by adjusting the diameter of the laser beam used to eject the material. Beads fabricated by this technique in our lab were consistently produced from as large as 500 μm to as small as 50 μm in diameter (Fig. 5.5). Further, bead spacing in printed arrays was accurate within 2% of the desired spacing. The viability of cells encapsulated within beads remained high, very close to what is found using gelatin-based planar LDW. This work leads into ongoing research that utilizes single microbeads as “voxels” to form larger 3D structures. Microbeads are printed consecutively, LbL, on top of one another, to produce an overall 3D construct in which the architecture and composition are prescribed with microbead-level fidelity.

5.4.4 FABRICATION OF CORE-SHELLED MICROENVIRONMENTS

Microbeads have many uses for 3D cell-based applications. However, in certain circumstances where one would want the cells to produce their own scaffold material, a microcapsule may be a more ideal structure. A microcapsule is a core-shelled spherical microenvironment, made from processing a microbead (Orive, Tam, Pedraz, & Hallé, 2006). In the case of an alginate microbead (negatively charged polymer), a microcapsule is formed by the addition of an oppositely charged

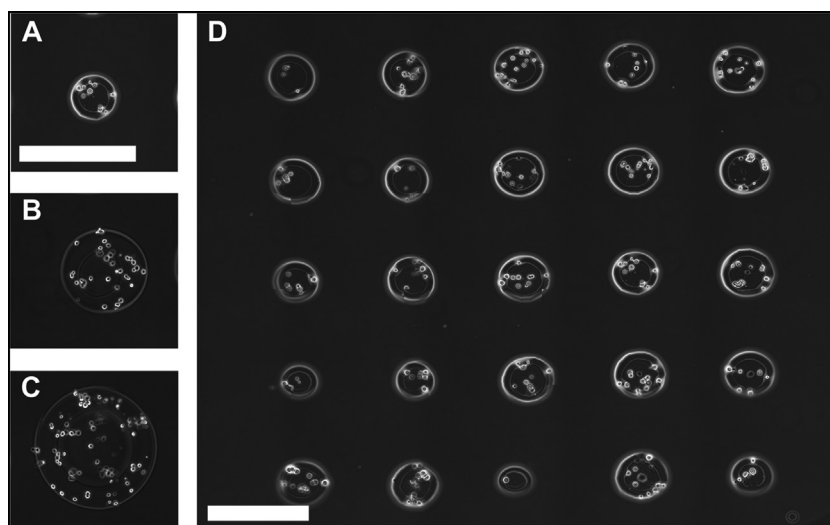


FIGURE 5.5

Representative images of LDW-fabricated microbeads encapsulating living cells (i.e., MDA-MB-231 tumor cells). Microbeads fabricated at different sizes: (A) 200- μm diameter, (B) 350- μm -diameter, and (C) 500- μm -diameter microbeads, illustrate the size control achieved by adjusting the beam diameter of the laser in LDW. (D) A printed pattern (5 $\times$ 5 array, with 600 μm centroid-to-centroid spacing) of size-controlled microbeads (200- μm -diameter) illustrating the spatial control of microbead LDW. Scale bars are 500 μm .

polyelectrolyte, such as poly-L-lysine or chitosan (positively charged polymers). The positively charged polymer complexes with the surface of the alginate microbead form a shell surrounding the bead. The bulk alginate in the microbead is then liquefied by the addition of a chelating agent to remove the divalent cation that cross-links the alginate. This results in a polyelectrolyte shell composed of the two oppositely charged polymers, encapsulating the cells in a liquid core.

A particularly promising application for LDW-patterned microcapsules is for creating size-controlled multicellular aggregates. With the fine control of beam diameter offered by LDW, capsules ranging from 100 to 500 μm have been fabricated, and this range can be extended for both smaller and larger capsules with changes to the beam-delivery optics. This range is well suited for *in vitro* models, such as multicellular tumor spheroids (MCTSs) and EBs, in which aggregate size/shape heavily influences model behavior and physiologic relevance. For example, LDW microbeads have been processed into chitosan-alginate microcapsules (Fig. 5.6A) to create size-controlled 3D cellular aggregates, that is, MCTSs and EBs (Fig. 5.6B and C), and which retain initial bead position on the patterned substrate (Kingsley, Roberge, et al., 2019). The advantage of these LDW-fabricated capsules is that they can be placed accurately, within 2% of their target spacing.

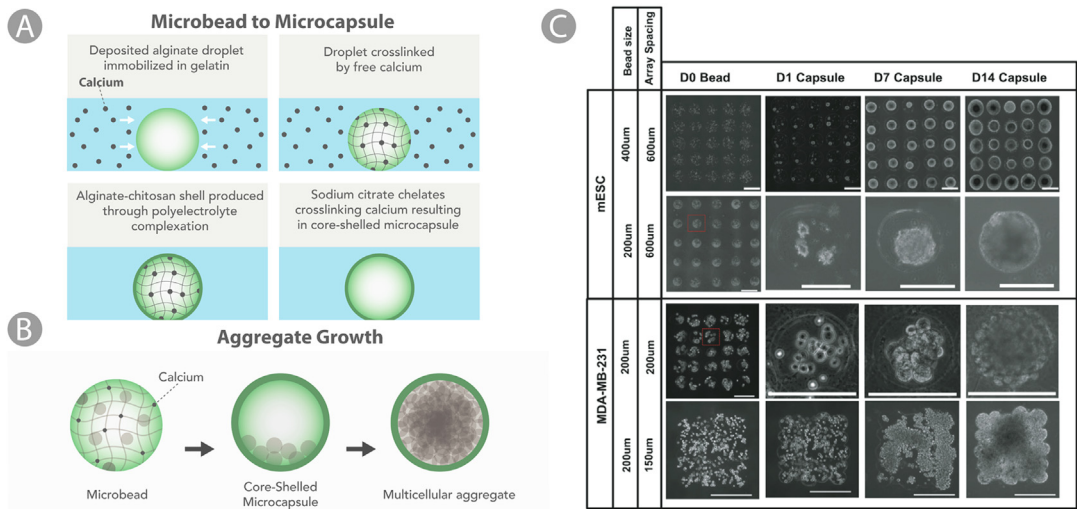


FIGURE 5.6

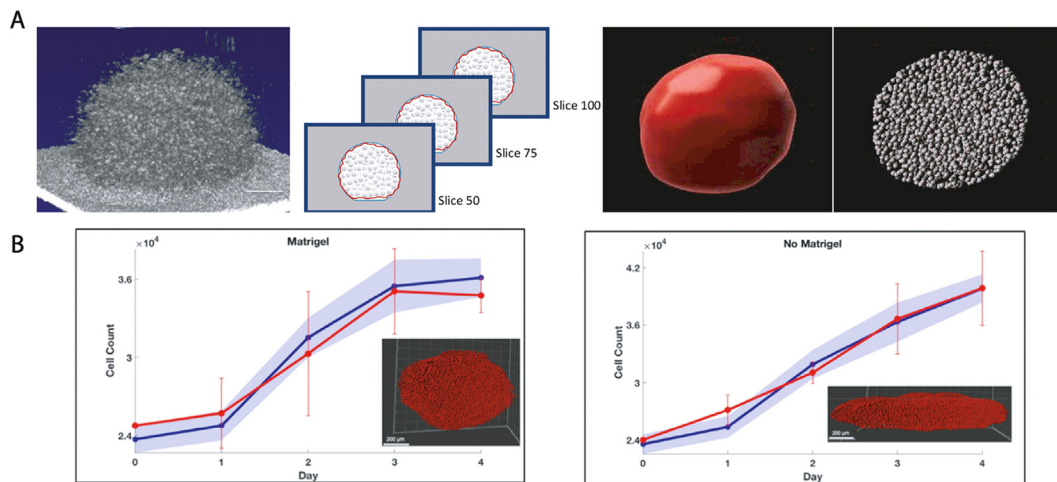
Microcapsules fabricated by processing microbeads printed by LDW. (A) Cell-suspending alginate is deposited onto a receiving substrate where it is rapidly crosslinked *in situ* and immobilized before processing into a core-shelled microcapsule. (B) Encapsulated cells proliferate within the contained environment to form cellular aggregates. (C) Human breast cancer cells and mouse embryonic stem cells in chitosan capsules from 1 to 14 days. This illustrates that the microcapsules allow cell growth over time within a constrained geometry: the breast cancer cells fill the hollow capsule to form spheroids, and the stem cells form an embryoid body (EB) contained within the microcapsule. The shape of these cell aggregates can be controlled by the core-shell structure geometry. Scale bars are 200 μm .

Adapted from Kingsley, Roberge, et al. (2019), with permission.

Leveraging LDW's microbead size control and spatial localization, it is possible to print cell-loaded beads in an overlapping fashion to fabricate larger continuous structures. These can be processed into custom core-shelled geometries to create size- and shape-controlled cellular aggregates, such as those formed within micromats (bottom row of Fig. 5.6C). Following printing, these cell-loaded mats were processed into core-shelled structures with a continuous geometry within which cells can proliferate, migrate, and aggregate. In this way, cell-loaded microbeads can be used as building blocks to form large 3D aggregates, with custom geometries and composition prescribed at the individual microbead scale.

Notably, LDW-based microcapsules have demonstrated the potential to form 3D cellular aggregates, even in cell lines which have little to no ability to self-aggregate. Such aggregates are highly desired for physiologically relevant *in vitro* models of those cell lines, yet many “gold standard” aggregate fabrication techniques (e.g., liquid overlay, hanging drop) have not demonstrated success in getting such cells to aggregate. Often, these cultures are supplemented with Matrigel to encourage aggregation (Badea et al., 2019; Froehlich et al., 2016; Nagelkerke, Bussink, Sweep, & Span, 2013; Roberge et al., 2020), and although aggregation is generally improved, doing so introduces the additional unknown of Matrigel's influence and its biologic/compositional variations. In preliminary studies with a representative nonaggregating cell line, AU565 HER2 + breast cancer cells, the addition of Matrigel to liquid overlay culture improved aggregation, yet was unable to promote sufficient aggregation to achieve the desired spherical morphology. Although these popular spheroid formation techniques promote cell–cell interaction over cell–substrate interaction to encourage aggregation, LDW-based microcapsules also constrain cell proliferation within a pre-defined spherical space, making them a particularly promising fabrication approach for cells that require extra stimuli to form aggregates. Indeed, some of our preliminary work has demonstrated the creation of AU565 tumor spheroids within LDW-microcapsules, with aggregates achieving sufficiently high volumes and sphericities for physiologically relevant behavior (data not shown). Additionally, these spheroids were formed without the addition of exogenous Matrigel, making them suitable for mechanistic investigations seeking to limit the effects of exogenous external influences (Baker, Banerjee, Bonin, & Guthold, 2016; van Tienderen, Koerkamp, Ijzermans, van der Laan, & Versteegen, 2019).

An *in vitro* 3D cell aggregate's ability to model/mimic important *in vivo* behaviors is strongly influenced by the aggregate size and shape. Hence, it is critical to quantitatively assess the 3D aggregate morphology to confirm its utility. Recently, a number of techniques to measure and track model morphology during development have emerged (Alessandri et al., 2013; Huang et al., 2017; Piccinini, Tesei, & Bevilacqua, 2016). One particularly promising approach employs Optical Coherence Tomography (OCT) structural imaging with Imaris image analysis software (Bitplane USA), which is capable of rendering spheroids within the collected 3D volume scans and measuring resultant morphology (Kingsley, McCleery, et al., 2019; Kingsley, Roberge, et al., 2019; Roberge et al., 2020). In this way, this technique can nondestructively analyze mesoscopic aggregates in 3D during their development, and in response to drug treatments. This technique has been applied to LDW microcapsule-based aggregates and confirmed that the aggregates formed within capsules reach spheroid status after ~14 days of culture for a variety of cell lines (Fig. 5.7A) (i.e., MDA-MB-231 triple negative breast cancer (Kingsley, Roberge, et al., 2019), CCE Mouse Embryonic Stem Cells (Kingsley, Roberge, et al., 2019)), AU565 HER2 + breast cancer cells (unpublished data). These aggregates were found to have high sphericities (>0.8) (Bellotti, Duchi, Bevilacqua, Lucarelli, & Piccinini, 2016; Noto et al., 2013) suitable for *in vitro* studies, and controlled volumes based on the size of the original

**FIGURE 5.7**

Analysis of MDA-MB-231 tumor spheroids with OCT. (A) OCT volume scan of large (400 μm diameter) spheroid within capsule on day 14 and Imaris image analysis of spheroid for morphology and cell density quantification. (B) Validation of OCT for cell counting in tumor aggregates (red) versus hemocytometry (blue shading).

Adapted from Roberge et al. (2020), with permission.

printed microbead, thus demonstrating LDW-based microcapsules' utility to induce spheroid formation. In addition to morphologic quantification, this OCT + Imaris technique has recently been extended for nondestructive and label-free quantification of cell number (Fig. 5.7B) and viability within 3D aggregates (Roberge et al., 2020). While this technique was demonstrated on aggregates fabricated via liquid overlay, it shows great promise for quantitatively monitoring cell number and viability in LDW-microcapsules, during spheroid development and in response to drug treatment; both critical metrics of interest for *in vitro* modeling.

5.5 CONCLUSIONS AND FUTURE DIRECTIONS

There are aspects of cellular behavior, for example, migration, differentiation, and certain types of gene expression that are heavily regulated by the cellular microenvironment. Both mechanical signals from the substrate and biochemical signals, either soluble or insoluble, have profound effects on cell fate and function, and are being widely studied. Cell–cell signaling is also an important influential factor in cell behavior, yet this is rarely studied, due, in part, to the complexity and technical challenges in doing so. Prescribing cell signaling within *in vitro* models requires precise control of cellular location and proximity within the fabricated microenvironment, which influences whether signaling is homotypic or heterotypic, the strength of the signal based on the number/density and placement of cells, and the signaling dynamics. Although it is all but impossible to mimic

the sheer complexity of multiple cell types and signals *in vivo*, spatially precise patterning within engineered substrates offers a powerful tool to study aspects of cellular signaling *in vitro*.

Of the cell printing techniques discussed herein, LDW is particularly well suited for applications that require spatial precision and controlled cell placement in engineered microenvironments. This technique has been used to print multiple types of cells in custom patterns, allowing fabrication of microenvironments that can maximize (or minimize) a desired behavior-based solely on cellular arrangement. Micropatterning has been used to show that cell size/shape or colony size can influence cell fates, but it generally does not allow evolution of structure. Moreover, the adhesive proteins that are patterned confound cell signaling, making it difficult to decouple the effect of the adhesive protein and the effect of cellular signaling.

For 3D bioprinting approaches, in order to get sufficient height to the structure, fairly robust materials such as hydrogels are often used to ensure both cell viability within the printed construct and 3D structural integrity. As discussed, LDW is a versatile printing technique that can be used with a wide variety of printed hydrogels (e.g., gelatin, Matrigel, alginate) as well as having great potential for a variety of potential substrates (e.g., hydrogels, electrospun fibers, microfluidic devices, and even living tissues). As with all applications encapsulating cells in 3D hydrogels constructs, a major limitation is the cells' ability to migrate and proliferate within these environments, which require degradation and ECM replacement. 3D cellular constructs, particularly thick constructs such as those developed through LbL approaches, may take weeks or months to become fully cellularized, because of the geometric restriction of nanoporous hydrogels. To overcome this limitation, future applications in complex *in vitro* 3D tissue models should incorporate vasculature, lamina, and other structures to help facilitate nutrient/waste exchange and overcome present restrictions. Additionally, continued exploration into functionalized hydrogels for printing (e.g., PEG, GelMA) may better facilitate cell growth and migration while also incorporating critical porosity.

While LDW offers capabilities for generating spatially precise 2D cellular microenvironments, it has even more power beyond micropatterning approaches in fabricating 3D microenvironments. Similar to inkjet printing, LDW has demonstrated the ability to print cells and biomaterials in a LbL manner. Although the throughput of LDW is lower than that of inkjet, it offers higher resolution, potentially allowing controlled studies of 3D cell–cell interactions in complex geometries. Our ongoing studies in LbL LDW printing are seeking to fabricate thick, heterotypic *in vitro* microenvironments with cell placement informed by histologic information, wherein multiple cell types are precisely placed in accordance with their spatial distributions in consecutive histologic slices.

Another recent method for creating 3D microenvironments using LDW is the fabrication of 3D microbeads of controlled size and placement. In contrast to traditional methods for fabricating microbeads, LDW allows fabrication and placement in a single step, via *in situ* crosslinking of a hydrogel. This enables cells encapsulated in the microbead to be precisely placed in 3D microenvironments. As a result, this method allows the study of cells within a 3D microenvironment, but on a 2D substrate, which permits high-quality imaging and analysis. This feature also makes microbead printing compatible with planar (2D) LDW, so hybrid 2D/microbead constructs can be fabricated. Hybrid constructs allow 2D cellular studies based on point sources of material or factors delivered by beads. Encapsulated cells within beads can potentially deliver factors continuously, or beads themselves could be used for delivery. Beads can serve also as nodes at precise spatial locations to direct 2D spatial migration.

The true power of this technique is realized when additional processing of microbeads with a cationic polymer allows them to be shelled, and the hydrogel liquefied, leaving a capsule that

allows cellular migration and proliferation within the boundary of the capsule. These microcapsules enable the rapid formation of dense cellular constructs by eliminating the need for ECM degradation prior to proliferate. This technique has been demonstrated with a variety of cell lines to form cellular aggregates, even in cell lines with low self-aggregation capabilities. The morphology of matured capsule-based aggregates has been analyzed and shown to match the geometry of the original printed microbead pre-processing, showing great potential for the use of microbeads as building blocks to create larger microcapsule constructs with unique geometries.

The LDW field continues to advance in the direction of 3D patterning. 3D LbL and microbead printing approaches both hold promise for studying thicker, more physiologically-representative 3D cellular microenvironments, and allow a wide range of applications based on the same technology. The advances made in printed microcapsules shows great promise for growth of size- and shape-controlled cellular aggregates, as well as fabrication of larger cellular constructs with unique geometries. Indeed, the coming decade holds great promise for the advancement of LDW for cellular studies in tissue engineering and regenerative medicine based on precisely controlled 2D and 3D microenvironments.

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QUESTIONS

Q1. In what instances is it desirable to control cell placement when creating engineered microenvironments, and why is it desirable? In particular, how can spacing between cells or populations of cells influence the biologic response?

Q2. Consider the trade-offs between each of the three patterning techniques discussed—inkjet bioprinting, micropatterning, and LDW bioprinting. For each technique provide an example application where it would be the best option, and briefly explain why it would be preferred over the other two techniques. Explain how printing live cells limits the choice of bioink material compared to printing without suspended cells.

Q3. What is the role of a sacrificial layer in LDW bioprinting, and what are key features of a material that would make it a good candidate for use as a sacrificial layer?

Q4. Cell printing via LDW was initially established utilizing Matrigel on both the print ribbon and the receiving substrate. More recently, a gelatin-based LDW technique was developed as an alternative.

- (a) Why would it be *biologically* desirable to remove Matrigel from the process?
- (b) What unique function does gelatin provide when utilized on the print ribbon?
- (c) What unique function does gelatin provide when used on the receiving substrate?

Q5. Cell behavior can be greatly influenced by the dimension of its ECM. The same cell could respond quite differently in 2D and 3D microenvironments. List (1) examples of how cells sense and respond differently when cultured on a planar substrate (2D), or within a 3D hydrogel, and (2) some important properties of a 3D hydrogel microenvironment that can influence the cellular migration within it.

Q6. What are the differences between LDW microbead patterning and using a mold to generate a hydrogel in the desired shape? Provide ways in which the unique fabrication capabilities of LDW microbead patterning can be used to create custom 3D microenvironments and engineered cellular constructs.

References

- Agarwal, A., Goss, J. A., Cho, A., McCain, M. L., & Parker, K. K. (2013). Microfluidic heart on a chip for higher throughput pharmacological studies. *Lab on a Chip*, 13(18), 3599–3608.
- Aisenbrey, E., & Murphy, W. (2020). Synthetic alternatives to Matrigel. *Nature Reviews Materials*, 5(7), 539–551.
- Alessandri, K., Sarangi, B. R., Gurchenkov, V. V., Sinha, B., Kießling, T. R., Fetler, L., . . . Nassoy, P. (2013). Cellular capsules as a tool for multicellular spheroid production and for investigating the mechanics of tumor progression *in vitro*. *Proceedings of the National Academy of Sciences*, 110(37), 14843–14848.
- Amsden, B. G., & Goosen, M. F. A. (1997). An examination of factors affecting the size, distribution, and release characteristics of polymer microbeads made using electrostatics. *Journal of Controlled Release*, 43, 183–196.
- Anseth, K., & Lin, C. (2009). PEG hydrogels for the controlled release of biomolecules in regenerative medicine. *Pharmaceutical Research*, 26(3), 631–643.
- Badea, M. A., Balas, M., Hermenean, A., Ciceu, A., Herman, H., Ionita, D., & Dinischiotu, A. (2019). Influence of matrigel on single- and multiple-spheroid cultures in breast cancer research. *SLAS Discovery*, 24(5), 563–578.
- Baker, S. R., Banerjee, S., Bonin, K., & Guthold, M. (2016). Determining the mechanical properties of electrospun poly- ϵ -caprolactone (PCL) nanofibers using AFM and a novel fiber anchoring technique. *Materials Science and Engineering C*, 59, 203–212.
- Banks, D. P., Kaur, K., Gazia, R., Fardel, R., Nagel, M., Lippert, T., & Eason, R. W. (2008). Triazene photopolymer dynamic release layer-assisted femtosecond laser-induced forward transfer with an active carrier substrate. *EPL (Europhysics Letter)*, 83, 38003.
- Barron, J. A., Krizman, D. B., & Ringeisen, B. R. (2005). Laser printing of single cells: Statistical analysis, cell viability, and stress. *Biomedical Engineering*, 33, 121–130.
- Barron, J. A., Ringeisen, B. R., Kim, H., Spargo, B. J., & Chrisey, D. B. (2004a). Application of laser printing to mammalian cells. *Thin Solid Films*, 453, 383–387.
- Barron, J. A., Wu, P., Ladouceur, H. D., & Ringeisen, B. R. (2004b). Biological laser printing: A novel technique for creating heterogeneous 3-dimensional cell patterns. *Biomedical Microdevices*, 6, 139–147.
- Bauwens, C. L., Peerani, R., Niebruegge, S., Woodhouse, K. A., Kumacheva, E., Husain, M., & Zandstra, P. W. (2008). Control of human embryonic stem cell colony and aggregate size heterogeneity influences differentiation trajectories. *Stem Cells*, 26, 2300–2310.
- Bavli, D., Prill, S., Ezra, E., Levy, G., Cohen, M., Vinken, M., . . . Nahmias, Y. (2016). Real-time monitoring of metabolic function in liver-on-chip microdevices tracks the dynamics of Mitochondrial dysfunction. *Proceedings of the National Academy of Sciences of the United States of America*, 113(16), E2231–E2240.

- Bellotti, C., Duchi, S., Bevilacqua, A., Lucarelli, E., & Piccinini, F. (2016). Long term morphological characterization of mesenchymal stromal cells 3D spheroids built with a rapid method based on entry-level equipment. *Cytotechnology*, 68(6), 2479–2490.
- Berrier, A., & Yamada, K. (2007). Cell–matrix adhesion. *Journal of Cellular Physiology*, 213, 565–573.
- Boland, T., Xu, T., Damon, B., & Cui, X. (2006). Application of inkjet printing to tissue engineering. *Biotechnology Journal*, 1, 910–917.
- Calvert, P. (2001). Inkjet printing for materials and devices. *Chemistry of Materials*, 13, 3299–3305.
- Catros, S., Guillemot, F., Nandakumar, A., Ziane, S., Moroni, L., Habibovic, P., ... Fricain, J.-C. (2011). Layer-by-layer tissue microfabrication supports cell proliferation *in vitro* and *in vivo*. *Tissue Engineering Part C: Methods*, 18, 62–70.
- Chaudhuri, O., Gu, L., Darnell, M., Klumpers, D., Bencherif, S. A., Weaver, J. C., ... Mooney, D. J. (2015). Substrate stress relaxation regulates cell spreading. *Nature Communications*, 6, 6364.
- Chen, C., Barron, J., & Ringeisen, B. (2006). Cell patterning without chemical surface modification: Cell-cell interactions between printed bovine aortic endothelial cells (BAEC) on a homogeneous cell-adherent hydrogel. *Applied Surface Science*, 252, 8641–8645.
- Chen, C. S., Mrksich, M., Huang, S., Whitesides, G. M., & Ingber, D. E. (1998). Micropatterned surfaces for control of cell shape, position, and function. *Biotechnology Progress*, 14, 356–363.
- Chrissey, D. B., Pique, A., Fitz-Gerald, J., Auyeung, R. C. Y., McGill, R. A., Wu, H. D., & Duignan, M. (2000). New approach to laser direct writing active and passive mesoscopic circuit elements. *Applied Surface Science*, 154–155, 593–600.
- Colina, M., Serra, P., Fernández-Pradas, J. M., Sevilla, L., & Morenza, J. L. (2005). DNA deposition through laser induced forward transfer. *Biosensors & Bioelectronics*, 20, 1638–1642.
- Constantini, M., Testa, S., Mozetic, P., Barbetta, A., Fuoco, C., Fornetti, E., ... Gargioli, C. (2017). Microfluidic-enhanced 3D bioprinting of aligned myoblast-laden hydrogels leads to functionally organized myofibers *in vitro* and *in vivo*. *Biomaterials*, 131, 98–110.
- Desmarais, S. M., Haagsman, H. P., & Barron, A. E. (2012). Microfabricated devices for biomolecule encapsulation. *Electrophoresis*, 33, 2639–2649.
- Dias, A. D., Unser, A. M., Xie, Y., Chrissey, D. B., & Corr, D. T. (2014). Generating size-controlled embryoid bodies using laser direct-write. *Biofabrication*, 6, 025007.
- Dinca, V., Farsari, M., Kafetzopoulos, D., Popescu, A., Dinescu, M., & Fotakis, C. (2008). Patterning parameters for biomolecules microarrays constructed with nanosecond and femtosecond UV lasers. *Thin Solid Films*, 516, 6504–6511.
- Doraiswamy, A., Narayan, R. J., Harris, M. L., Qadri, S. B., Modi, R., Chrissey, D. B., ... Carolina, N. (2006a). Laser microfabrication of hydroxyapatite-osteoblast-like cell composites. *Journal of Biomedical Materials Research*, 80, 635–643.
- Doraiswamy, A., Narayan, R., Lippert, T., Urech, L., Wokaun, A., Nagel, M., ... Auyeung, R. (2006). Excimer laser forward transfer of mammalian cells using a novel triazine absorbing layer. *Applied Surface Science*, 252, 4743–4747.
- Engler, A., Sen, S., Sweeney, H., & Discher, D. (2006). Matrix elasticity directs stem cell lineage specification. *Cell*, 126, 677–689.
- Fernández-Pradas, J. M., Colina, M., Serra, P., Dominguez, J., & Morenza, J. L. (2004). Laser-induced forward transfer of biomolecules. *Thin Solid Films*, 453, 27–30.
- Freytes, D. O., Wan, L. Q., & Vunjak-Novakovic, G. (2009). Geometry and force control of cell function. *Journal of Cellular Biochemistry*, 108, 1047–1058.
- Froehlich, K., Haeger, J., Heger, J., Pastuschek, J., Photini, S. M., Yan, Y., ... Schmidt, A. (2016). Generation of multicellular breast cancer tumor spheroids: Comparison of different protocols. *Journal of Mammary Gland Biology and Neoplasia*, 21, 89–98.

- Gaebel, R., Ma, N., Liu, J., Guan, J., Koch, L., Klopsch, C., ... Steinhoff, G. (2011). Patterning human stem cells and endothelial cells with laser printing for cardiac regeneration. *Biomaterials*, 32, 9218–9230.
- Godier, A. F. G., Marolt, D., Gerecht, S., Tajnsek, U., Martens, T. P., & Vunjak-Novakovic, G. (2008). Engineered microenvironments for human stem cells. *Birth Defects Research. Part C, Embryo Today: Reviews*, 84, 335–347.
- Gonzalez-Macia, L., Morrin, A., Smyth, M. R., & Killard, A. J. (2010). Advanced printing and deposition methodologies for the fabrication of biosensors and biodevices. *Analyst*, 135, 845–867.
- Gruene, M., Pflaum, M., Hess, C., Diamantouros, S., Schlie, S., Deiwick, A., ... Chichkov, B. (2011a). Laser printing of three-dimensional multicellular arrays for studies of cell-cell and cell-environment interactions. *Tissue Engineering Part C: Methods*, 17, 973–982.
- Gruene, M., Deiwick, A., Koch, L., Schlie, S., Unger, C., ... Chichkov, B. (2011b). Laser printing of stem cells for biofabrication scaffold-free autologous grafts. *Tissue Engineering Part C: Methods*, 17, 79–87.
- Gruene, M., Pflaum, M., Deiwick, A., Koch, L., Schlie, S., Unger, C., ... Chichkov, B. (2011c). Adipogenic differentiation of laser-printed 3D tissue grafts consisting of human adipose-derived stem cells. *Biofabrication*, 3, 015005.
- Guillemot, F., Souquet, A., Catros, S., Guillotin, B., Lopez, J., Faucon, M., ... Amédée, J. (2010). High-throughput laser printing of cells and biomaterials for tissue engineering. *Acta Biomaterialia*, 6, 2494–2500.
- Hopp, B., Smausz, T., Kresz, N., Barna, N., Bor, Z., Kolozsvári, L., ... Nógrádi, A. (2005). Survival and proliferative ability of various living cell types after laser-induced forward transfer. *Tissue Engineering*, 11, 1817–1823.
- Huang, Y., Wang, S., Guo, Q., Kessel, S., Rubinoff, I., Chan, L. L. Y., ... Zhou, C. (2017). Optical coherence tomography detects necrotic regions and volumetrically quantifies multicellular tumor spheroids. *Cancer Research*, 77(21), 6011–6020.
- Huebsch, N., Arany, P. R., Mao, A. S., Shvartsman, D., Ali, O. A., Bencherif, S. A., ... Mooney, D. J. (2010). Harnessing traction-mediated manipulation of the cell/matrix interface to control stem-cell fate. *Nature Materials*, 9, 518–526.
- Huh, D., Matthews, B. D., Mammoto, A., Montoya-Zavala, M., Yuan Hsin, H., & Ingber, D. E. (2010). Reconstituting organ-level lung functions on a chip. *Science (New York, N.Y.)*, 328(5986), 1662–1668.
- Hui, E., & Bhatia, S. (2007). Micromechanical control of cell-cell interactions. *Proceedings of the National Academy of Sciences*, 104, 5722–5726.
- Jackman, R. J., Wilbur, J. L., & Whitesides, G. M. (1995). Fabrication of submicrometer features on curved substrates by microcontact printing. *Science (New York, N.Y.)*, 269, 664–666.
- Jang, K.-J., Mehr, A. P., Hamilton, G. A., McPartlin, L. A., Chung, S., Suh, K.-Y., & Ingber, D. E. (2013). Human kidney proximal tubule-on-a-chip for drug transport and nephrotoxicity assessment. *Integrative Biology*, 5(9), 1119–1129.
- Kaji, H., Camci-Unal, G., Langer, R., & Khademhosseini, A. (2011). Engineering systems for the generation of patterned cocultures for controlling cell-cell interactions. *Biochimica et Biophysica Acta*, 1810, 239–250.
- Kattamis, N. T., Purnick, P. E., Weiss, R., & Arnold, C. B. (2007). Thick film laser induced forward transfer for deposition of thermally and mechanically sensitive materials. *Applied Physics Letters*, 91, 171120.
- Keriquel, V., Oliveira, H., Rémy, M., Ziane, S., Delmond, S., Rousseau, B., ... Fricain, J.-C. (2017). *In situ* printing of mesenchymal stromal cells, by laser-assisted bioprinting, for *in vivo* bone regeneration applications. *Scientific Reports*, 7(1), 1–10.
- Kim, H. J., Huh, D., Hamilton, G., & Ingber, D. E. (2012). Human gut-on-a-chip inhabited by microbial flora that experiences intestinal peristalsis-like motions and flow. *Lab on a Chip*, 12(12), 2165–2174.

- Kingsley, D. M., Dias, A. D., Chrisey, D. B., & Corr, D. T. (2013). Single-step laser-based fabrication and patterning of cell-encapsulated alginate microbeads. *Biofabrication*, 5, 045006.
- Kingsley, D. M., Dias, A. D., & Corr, D. T., (2016). Microcapsules and 3D customizable shelled microenvironments from laser direct-written microbeads. *Biotechnology and bioengineering*, 113, 2264–2274.
- Kingsley, D. M., McCleery, C. H., Johnson, C. D., Bramson, M. T., Rende, D., Gilbert, R. J., & Corr, D. T. (2019). Multi-modal characterization of polymeric gels to determine the influence of testing method on observed elastic modulus. *Journal of the Mechanical Behavior of Biomedical Materials*, 92, 152–161.
- Kingsley, D. M., Roberge, C. L., Rudkouskaya, A., Faulkner, D. E., Barroso, M., Intes, X., & Corr, D. T. (2019). Laser-based 3D bioprinting for spatial and size control of tumor spheroids and embryoid bodies. *Acta Biomaterialia*, 95, 357–370.
- Kleinman, H. K., & Martin, G. R. (2005). Matrigel: Basement membrane matrix with biological activity. *Seminars in Cancer Biology*, 15, 378–386.
- Kleinman, H. K., McGarvey, M. L., Hassell, J. R., Star, V. L., Cannon, F. B., Laurie, G. W., & Martin, G. R. (1986). Basement membrane complexes with biological activity. *Biochemistry*, 25, 312–318.
- Koch, L., Brandt, O., Deiwick, A., & Chichkov, B. (2017). Laser-assisted bioprinting at different wavelengths and pulse durations with a metal dynamic release layer: A parametric study. *International Journal of Bioprinting*, 3, 42–53.
- Koch, L., Deiwick, A., Schlie, S., Michael, S., Gruene, M., Coger, V., . . . Chichkov, B. (2012). Skin tissue generation by laser cell printing. *Biotechnology and Bioengineering*, 109, 1855–1863.
- Koch, L., Kuhn, S., Sorg, H., Gruene, M., Schlie, S., Gaebel, R., . . . Chichkov, B. (2010). Laser printing of skin cells and human stem cells. *Tissue Engineering Part C: Methods*, 16, 847–854.
- Lee, L. H., Peerani, R., Ungrin, M., Joshi, C., Kumacheva, E., & Zandstra, P. W. (2009). Micropatterning of human embryonic stem cells dissects the mesoderm and endoderm lineages. *Stem Cell Research*, 2, 155–162.
- Lee, W., Debasitis, J. C., Lee, V. K., Lee, J.-H., Fischer, K., Edminster, K., . . . Yoo, S.-S. (2009). Multi-layered culture of human skin fibroblasts and keratinocytes through three-dimensional freeform fabrication. *Biomaterials*, 30, 1587–1595.
- Liu, W., Heinrich, M. A., Zhou, Y., Akpek, A., Hu, N., Liu, X., . . . Zhang, Y. S. (2017). Extrusion bioprinting of shear-thinning gelatin methacryloyl bioinks. *Advanced Healthcare Materials*, 6(12), 1601451.
- Liu, Y., Wang, X., Kaufman, D. S., & Shen, W. (2011). A synthetic substrate to support early mesodermal differentiation of human embryonic stem cells. *Biomaterials*, 32, 8058–8066.
- Loessner, D., Meinert, C., Kaemmerer, E., Martine, L. C., Yue, K., Levett, P. A., . . . Hutmacher, D. W. (2016). Functionalization, preparation, and use of cell-laden gelatin methacryloyl-based hydrogels as a modular tissue culture platforms. *Nature Protocols*, 11(4), 727.
- Lund, A. W., Yener, B., Stegmann, J. P., & Plopper, G. E. (2009). The natural and engineered 3D microenvironment as a regulatory cue during stem cell fate determination. *Tissue Engineering Part B: Reviews*, 15, 371–380.
- Massia, S. P., & Hubbell, J. A. (1991). An RGD spacing of 440 nm is sufficient for integrin alpha V beta 3-mediated fibroblast spreading and 140 nm for focal contact and stress fiber formation. *The Journal of Cell Biology*, 114, 1089–1100.
- Meshel, A. S., Wei, Q., Adelstein, R. S., & Sheetz, M. P. (2005). Basic mechanism of three-dimensional collagen fibre transport by fibroblasts. *Nature Cell Biology*, 7, 157–164.
- Nagelkerke, A., Bussink, J., Sweep, F. C. G. J., & Span, P. N. (2013). Generation of multicellular tumor spheroids of breast cancer cells: How to go three-dimensional. *Analytical Biochemistry*, 437(1), 17–19.
- Noto, A., Raffa, S., De Vitis, C., Roscilli, G., Malpicci, D., Coluccia, P., . . . Mancini, R. (2013). Stearoyl-CoA desaturase-1 is a key factor for lung cancer-initiating cells. *Cell Death and Disease*, 4(12), e947-11.
- Odde, D. J., & Renn, M. J. (2000). Laser-guided direct writing of living cells. *Biotechnology and Bioengineering*, 67, 312–318.

- Oleaga, C., Bernabini, C., Smith, A. S. T., Srinivasan, B., Jackson, M., McLamb, W., ... Hickman, J. J. (2016). Multi-organ toxicity demonstration in a functional human *in vitro* system composed of four organs. *Scientific Reports*, 6(1), 1–17.
- Orive, G., Tam, S. K., Pedraz, J. L., & Hallé, J.-P. (2006). Biocompatibility of alginate-poly-L-lysine microcapsules for cell therapy. *Biomaterials*, 27, 3691–3700.
- Ouyang, L., Armstrong, J. P., Lin, Y., Wojciechowski, J. P., Lee-Reeves, C., Hachim, D., ... Stevens, M. M. (2020). Expanding and optimizing 3D bioprinting capabilities using complementary network bioinks. *Science Advances*, 1(6), eabc5529.
- Ovsianikov, A., Gruene, M., Pflaum, M., Koch, L., Maiorana, F., Wilhelmi, M., ... Chichkov, B. (2010). Laser printing of cells into 3D scaffolds. *Biofabrication*, 2, 014104.
- Pampaloni, F., Reynaud, E. G., & Stelzer, E. H. K. (2007). The third dimension bridges the gap between cell culture and live tissue. *Nature Reviews. Molecular Cell Biology*, 8, 839–845.
- Patz, T. M., Doraiswamy, A., Narayan, R. J., He, W., Zhong, Y., Bellamkonda, R., ... Chrisey, D. B. (2005). Three-dimensional direct writing of B35 neuronal cells. *Journal of Biomedical Materials Research, B: Applied Biomaterials*, 78, 124–130.
- Pedersen, J., & Swartz, M. (2005). Mechanobiology in the third dimension. *Annals of Biomedical Engineering*, 33, 1469–1490.
- Peerani, R., Rao, B. M., Bauwens, C., Yin, T., Wood, G. A., Nagy, A., ... Zandstra, P. W. (2007). Niche-mediated control of human embryonic stem cell self-renewal and differentiation. *The EMBO Journal*, 26, 4744–4755.
- Petrie, T. A., Capadona, J. R., Reyes, C. D., & García, A. J. (2006). Integrin specificity and enhanced cellular activities associated with surfaces presenting a recombinant fibronectin fragment compared to RGD supports. *Biomaterials*, 27, 5459–5470.
- Pham, Q. P., Sharma, U., & Mikos, A. G. (2006). Electrospinning of polymeric nanofibers for tissue engineering applications: A review. *Tissue Engineering*, 12, 1197–1211.
- Phamduy, T., Sweat, R. S., Azimi, M. S., Burrow, M. E., Murfee, W. L., & Chrisey, D. B. (2015). Printing cancer cells into intact microvascular networks: A model for investigating cancer cell dynamics during angiogenesis. *Integrative Biology*, 7(9), 1068–1078.
- Piccinini, F., Tesei, A., & Bevilacqua, A. (2016). Single-image based methods used for non-invasive volume estimation of cancer spheroids: A practical assessing approach based on entry-level equipment. *Computer Methods and Programs in Biomedicine*, 135, 51–60.
- Piner, R., Zhu, J., Xu, F., Hong, S., & Mirkin, C. (1999). “Dip-pen” nanolithography. *Science (New York, N. Y.)*, 283, 661–663.
- Pirlo, R. K., Dean, D. M. D., Knapp, D. R., & Gao, B. Z. (2006). Cell deposition system based on laser guidance. *Biotechnology Journal*, 1, 1007–1013.
- Raof, N. A., Schiele, N. R., Xie, Y., Chrisey, D. B., & Corr, D. T. (2011). The maintenance of pluripotency following laser direct-write of mouse embryonic stem cells. *Biomaterials*, 32, 1802–1808.
- Ringeisen, B. R., Wu, P. K., Kim, H., Piqué, A., Auyeung, R. Y. C., Young, H. D., ... Krizman, D. B. (2002a). Picoliter-scale protein microarrays by laser direct write. *Chemical Biotechnology in Progress*, 18, 1126–1129.
- Ringeisen, B., Chrisey, D., Krizman, D., Kim, H., Young, P., & Spargo, B. (2002b). Cell-by-cell construction of living tissue by ambient laser transfer. *2nd annual international IEEE-EMBS special topic conference on microtechnologies in medicine & biology*, 120–125.
- Ringeisen, B. R., Barron, J. A., Young, D., Othon, C. M., Wu, P. K., Ladoucuer, D., & Spargo, B. J. (2008). *Laser printing cells. Virtual prototyping & bio manufacturing in medical applications* (pp. 207–228). Springer.
- Ringeisen, B. R., Kim, H., Barron, J. A., Krizman, D. B., Chrisey, D. B., Jackman, S., ... Spargo, B. J. (2004). Laser printing of pluripotent embryonal carcinoma cells. *Tissue Engineering*, 10, 483–491.

- Roberge, C. L., Kingsley, D. M., Faulkner, D. E., Sloat, C. J., Wang, L., Barroso, M., . . . Corr, D. T. (2020). Non-destructive tumor aggregate morphology and viability quantification at cellular resolution, during development and in response to drug. *Acta Biomaterialia*, 117, 322–334.
- Roth, E. A., Xu, T., Das, M., Gregory, C., Hickman, J. J., & Boland, T. (2004). Inkjet printing for high-throughput cell patterning. *Biomaterials*, 25, 3707–3715.
- Saunders, R. E., Gough, J. E., & Derby, B. (2008). Delivery of human fibroblast cells by piezoelectric drop-on-demand inkjet printing. *Biomaterials*, 29, 193–203.
- Schaub, N. J., Britton, T., Rajachar, R., & Gilbert, R. J. (2013). Engineered nanotopography on electrospun PLLA micro fibers modifies RAW 264.7 cell response. *ACS Applied Materials & Interfaces*, 5, 10173–10184.
- Schiele, N. R., Chrisey, D. B., & Corr, D. T. (2011). Gelatin-based laser direct-write technique for the precise spatial patterning of cells. *Tissue Engineering Part C: Methods*, 17, 289–298.
- Schiele, N. R., Corr, D. T., Huang, Y., Raof, N. A., Xie, Y., & Chrisey, D. B. (2010). Laser-based direct-write techniques for cell printing. *Biofabrication*, 2, 032001.
- Schiele, N. R., Koppes, R. A., Corr, D. T., Ellison, K. S., Thompson, D. M., Ligon, L. A., . . . Chrisey, D. B. (2009). Laser direct writing of combinatorial libraries of idealized cellular constructs: Biomedical applications. *Applied Surface Science*, 255, 5444–5447.
- Skardal, A., Murphy, S. V., Devarasetty, M., Mead, I., Kang, H. W., Seol, Y. J., . . . Atala, A. (2017). Multi-tissue interactions in an integrated three-tissue organ-on-a-chip platform. *Scientific Reports*, 7(1), 1–16.
- Sorkio, A., Koch, L., Koivusalo, L., Deiwick, A., Miettinen, S., Chichkov, B. & Skottman, H., (2018). Human stem cell based corneal tissue mimicking structures using laser-assisted 3D bioprinting and functional bioinks. *Biomaterials*, 171, 57–71.
- Tan, H., DeFail, A. J., Rubin, J. P., Chu, C. R., & Marra, K. G. (2010). Novel multiarm PEG-based hydrogels for tissue engineering. *Journal of Biomedical Materials Research. Part A*, 298(6), 699–703.
- Tasoglu, S., & Demirci, U. (2013). Bioprinting for stem cell research. *Trends in Biotechnology*, 31, 10–19.
- Tien, J., Nelson, C. M., & Chen, C. S. (2002). Fabrication of aligned microstructures with a single elastomeric stamp. *Proceedings of the National Academy of Sciences*, 99, 1758–1762.
- Toepke, M., Impellitteri, N., Theisen, J., & Murphy, W. (2012). Characterization of thiol-ene crosslinked PEG hydrogels. *Macromolecular Materials and Engineering*, 298(6), 699–703.
- Tse, J. R., & Engler, A. J. (2010). Preparation of hydrogel substrates with tunable mechanical properties. *Current Protocols in Cell Biology*, 47(1), 10–16.
- van der Helm, M. W., van der Meer, A. D., Eijkel, J. C. T., van den Berg, A., & Segerink, L. I. (2016). Microfluidic organ-on-chip technology for blood-brain barrier research. *Tissue Barriers*, 4(1), e1142493.
- van Tienderen, G. S., Koerkamp, B. G., Ijzermans, J. N. M., van der Laan, L. J. W., & Verstegen, M. M. A. (2019). Recreating tumour complexity in a dish: Organoid models to study liver cancer cells and their extracellular environment. *Cancers*, 11(11), 1706.
- Vinson, B. T., Phamduy, T. B., Shipman, J., Riggs, B., Strong, A. L., Sklare, S. C., . . . Chrisey, D. B., (2017). Laser direct-write based fabrication of a spatially-defined, biomimetic construct as a potential model for breast cancer cell invasion into adipose tissue. *Biofabrication*, 9, 025013.
- Wu, P. K., & Ringeisen, B. R. (2010). Development of human umbilical vein endothelial cell (HUVEC) and human umbilical vein smooth muscle cell (HUVSMC) branch/stem structures on hydrogel layers via biological laser printing (BioLP). *Biofabrication*, 2, 014111.
- Wu, P. K., Ringeisen, B. R., Callahan, J., Brooks, M., Bubb, D. M., Wu, H. D., . . . Chrisey, D. B. (2001). Deposition, structure, pattern deposition, and activity of biomaterial thin-films by matrix-assisted pulsed-laser evaporation (MAPLE) and MAPLE direct write. *Thin Solid Films*, 398–399, 607–614.
- Wu, P. K., Ringeisen, B. R., Krizman, D. B., Frondoza, C. G., Brooks, M., Bubb, D. M., . . . Chrisey, D. B. (2003). Laser transfer of biomaterials: Matrix-assisted pulsed laser evaporation (MAPLE) and MAPLE direct write. *The Review of Scientific Instruments*, 74, 2546.

- Xie, X. N., Chung, H. J., Sow, C. H., & Wee, A. T. S. (2006). Nanoscale materials patterning and engineering by atomic force microscopy nanolithography. *Materials Science and Engineering R: Reports*, 54, 1–48.
- Xie, Y., & Castracane, J. (2009). High-voltage, electric field-driven micro/nanofabrication for polymeric drug delivery systems. *IEEE Engineering in Medicine and Biology Society*, 28, 23–30.
- Xin, S., Chimene, D., Garza, J. E., Gaharwar, A. K., & Alge, D. L. (2019). Clickable PEG hydrogel microspheres as building blocks for 3D bioprinting. *Biomaterials Science*, 7(3), 1179–1187.
- Xiong, R., Chai, W., & Huang, Y. (2016). Laser printing-enabled direct creation of cellular heterogeneity in lab-on-a-chip devices. *Lab on a Chip*, 19(9), 1644–1656.
- Xiong, R., Zhang, Z., Chai, W., Chrisey, D. B., & Huang, Y., 2017. Study of gelatin as an effective energy absorbing layer for laser bioprinting. *Biofabrication*, 9, 024103.
- Xu, C., Inokuma, M. S., Denham, J., Golds, K., Kundu, P., Gold, J. D., & Carpenter, M. K. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. *Nature Biotechnology*, 19, 971–974.
- Xu, T., Jin, J., Gregory, C., Hickman, J. J., & Boland, T. (2005). Inkjet printing of viable mammalian cells. *Biomaterials*, 26, 93–99.
- Yamato, M., Kwon, O., Hirose, M., Kikuchi, A., & Okano, T. (2001). Novel patterned cell coculture utilizing thermally responsive grafted polymer surfaces. *Journal of Biomedical Materials Research*, 55, 137–140.
- Yousaf, M. N., Houseman, B. T., & Mrksich, M. (2001). Using electroactive substrates to pattern the attachment of two different cell populations. *Proceedings of the National Academy of Sciences*, 98, 5992–5996.
- Yue, K., Trujillo-de Santiago, G., Alvarez, M. M., Tamayol, A., Annabi, N., & Khademhosseini, A. (2015). Synthesis, properties, and biomedical applications of gelatin methacryloyl (GelMA) hydrogels. *Biomaterials*, 73, 254–271.
- Zhai, X., Ruan, C., Ma, Y., Cheng, D., Wu, M., Liu, W., . . . Lu, W. W. (2018). 3D-Bioprinted osteoblast-laden nanocomposite hydrogel constructs with induced microenvironments promote cell viability, differentiation, and osteogenesis both *in vitro* and *in vivo*. *Advanced Science*, 5(3), 1700550.
- Zhang, C., Liu, D., Mathews, S. A., Graves, J., Schaefer, T. M., Gilbert, B. K., . . . Chrisey, D. B. (2003). Laser direct-write and its application in low temperature co-fired ceramic (LTCC) technology. *Microelectronic Engineering*, 70, 41–49.

Bioink Printability Methodologies for Cell-Based Extrusion Bioprinting

6

Joshua Copus, Sang Jin Lee and Anthony Atala

Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, Winston-Salem, NC, United States

6.1 INTRODUCTION

The demands for personalized, implantable bioengineered tissue construct continuously increased (Arenas-Herrera, Ko, Atala, & Yoo, 2013; Atala, Kasper, & Mikos, 2012; Berebichez-Fridman & Montero-Olvera, 2018; Malda et al., 2013; Sachs, Mollica, & Bruno, 2017). A key piece to this future is the manufacturing processes that will be utilized. The manufacturing process determines the materials available for use, the architectures which can be created, and the time and cost which go into the final product. Additive manufacturing or three-dimensional (3D) printing has now become a household name and is quickly shaping the industries it has been involved in. It has also garnered significant interest within tissue engineering applications. Anatomical structures are often abnormal, complex, and highly variable between patients and disease progressions, advantaging the flexibility of additive manufacturing relative to more traditional techniques such as machining and molding (Moroni et al., 2018). Many different types of additive manufacturing have been utilized by the tissue engineering field for use with biomaterials and cells, referred to as “bioprinting.” Among those, extrusion-based bioprinting can easily incorporate multiple material types, a strategy that is imperative if any regional differences in biomaterials, cell types, or signaling molecules are desired (Gao, Zhao, Yang, Fu, & He, 2018; Gillispie et al., 2019; Gopinathan & Noh, 2018; Kang et al., 2016; Kim, Yoo, & Lee, 2016; Moroni et al., 2018; Panwar & Tan, 2016).

For the extrusion-based bioprinting, first and foremost are the materials that are a formulation of cells suitable for processing by an automated biofabrication technology that may also contain biologically active components and biomaterials (Groll et al., 2018). These materials, known as bioinks, have several major requirements they must fulfill (Basu et al., 2018; Jungst, Smolan, Schacht, Scheibel, & Groll, 2016). Bioinks must be biologically stable and nontoxic to cells and minimize host immune responses if implanted. Cells must be suspended directly in the material which requires both a thin enough material to mix the cells homogeneously and a thick enough material to maintain that distribution and prevent settling of the cells. Cell encapsulation also necessitates bioinks to be primarily composed of water, and for this reason, hydrogels are used (Guvendiren, Molde, Soares, & Kohn, 2016). Bioinks must provide bioactivity/cell attachment sites to allow for cell survival, attachment, and proliferation. They must have appropriate mechanical properties, preferably matching that of the tissue application both in terms of driving cell behavior

and to withstand mechanical forces during handling and implantation (Chimene, Lennox, Kaunas, & Gaharwar, 2016). Appropriate swelling and degradation characteristics are also necessary. Nutrients such as glucose and oxygen must be able to diffuse through the material to allow for cell survival in the deepest portions of the construct (Chang, Boland, Williams, & Hoying, 2011). Lastly, bioinks should also serve to direct cellular behavior through the naturally derived polymers, proteins, and binding sites that are found in the extracellular matrix (ECM) of the target tissue (Ali et al., 2019; Ozbolat & Hospodiuk, 2016). These requirements are common to most tissue engineering applications. Specific to the 3D printing process, bioinks should fulfill the three basic requirements: relatively higher viscosity to maintain cell suspension homogeneously and to provide initial structural integrity; strong shear-thinning behavior to minimize shear stress-driven cell damage during the printing process; and rapid crosslinking process after printing to maintain the structural integrity overtime (Gao, Gillispie, et al., 2018; Holzl et al., 2016; Kim et al., 2016).

In this chapter, we focus on physical factors impacting the printability of hydrogel-based bioinks specific to the 3D bioprinting process. We outline the underlying mechanisms that can influence different aspects of printability, which can be utilized for cell-based extrusion bioprinting.

6.2 DEFINITION OF PRINTABILITY

There are many requirements of bioinks that are specific to the bioprinting process. These requirements capture the different aspects of bioinks “printability.” In the broadest sense, printability refers simply to the ability to be printed. In this chapter, the following definition will be considered (Lee, Gillispie, Prim, & Lee, 2020): “The ability of a material, when subjected to a certain set of *printing conditions*, to be printed in a way which results in *printing outcomes* which are desirable for a given application.”

The first aspect of printability a bioink must overcome is an extrusion from a micron-scale nozzle tip. This ability is sometimes referred to as a bioink’s “extrudability.” If too much force is used to extrude the material, cells will be permanently damaged via shear stress. If the material has poor extrudability it can also affect print times which will be longer as higher flowrates cannot be achieved as easily (Li, Tian, Zhu, Schreyer, & Chen, 2010). As a result, if a bioink requires too high of an extrusion force, it cannot be used. After extrusion, a bioink must also be deposited in a predictable manner. Researchers have used a filament classification system to describe the different types of filaments which bioinks can form. Bioinks that form droplets at their tips will spread dramatically and stick to themselves. Continuous filaments are desired, and these filaments can either be uniform or nonuniform. Smooth filament deposition is easily controlled by the bioprinter, but nonuniform filaments may deviate from the printing path and have variable cross sections across their length (Kirchmayer, Gorkin, & in het Panhuis, 2015).

Following bioink deposition, the desired shape must be maintained as multiple layers are deposited on top of it. This “shape fidelity” of bioinks limits the ultimate size and shapes a bioink is able to structurally accomplish (Lee, Ng, & Yeong, 2019; Mandrycky, Wang, Kim, & Kim, 2016). Lastly, different printing conditions (nozzle size, print speed, etc.) may be required depending on the application, further constraining the system. The similarity of a printed construct to the desired dimensions, as influenced by the printing conditions, can be referred to as “printing accuracy.”

The term printability has been used to describe each of these requirements individually (Holzl et al., 2016; Hospodiuk, Dey, Sosnoski, & Ozbolat, 2017; Kyle, Jessop, Al-Sabah, & Whitaker, 2017; Kyle, Jessop, Tarassoli, Al-Sabah, & Whitaker, 2018); however, a bioink is not able to be bioprinted if it fails in any of these areas. Therefore this chapter will consider each of these factors as singular aspects of printability.

6.2.1 CONSIDERATION ON NOVEL BIOINK DEVELOPMENT

With all the constraints and requirements placed upon bioinks, it should come as no surprise that the available bioinks remain a major limitation of bioprinting. Researchers would benefit tremendously from both improvements upon current bioinks and an expanded bioink palette (Malda et al., 2013). A number of hydrogels and hydrogel combinations have been proposed in the literature; however, each has significant room for improvement in at least one area and sometimes multiple areas (Panwar & Tan, 2016). Contributing to this difficulty, many of the requirements placed on a bioink are in direct opposition. In many cases, bioinks which hold their shape particularly well upon deposition also require the most pressure to extrude (Malda et al., 2013). This tradeoff can be partially circumvented by various strategies including rapid postprinting crosslinking or gelation, the use of support baths, and bioinks which demonstrate particularly high degrees of shear thinning. Additionally, while great strides are frequently made on the bioactivity and tissue-specific potential of bioinks, these bioinks typically are not well suited for bioprinting and *vice versa*. For example, Pluronic F127 offers excellent printability for extrusion bioprinting but it is limited by its biological properties. Meanwhile, decellularized ECM (dECM) has been converted for use as a bioink due to its high, tissue-specific biological activity, but forms a very weak gel that cannot hold its shape on its own when bioprinted which must be compensated for with complementary strategies (Lee et al., 2019).

Bioink development is a growing field with the main objective of overcoming these various limitations. With respect to printability, bioink development itself is limited by its understanding of the underlying mechanisms which impact printability (Jungst et al., 2016). The factors that influence printability are numerous, complex, and interrelated (Guvendiren et al., 2016). Many relationships between printing conditions, material properties, and printability have been identified. However, many of these findings have been ambiguous or even conflicting and many more relationships remain unstudied. During the bioink development process, this knowledge is used to design, optimize, and improve a bioink (Chimene et al., 2016). Where it lacks, researchers must work using trial and error and move forward using less than ideal outcomes. The purpose of this chapter will be to examine these various relationships in an attempt to promote a better understanding and more holistic view of printability and the factors which influence it, in turn improving the bioink development process and therefore the bioinks available for use in bioprinting.

6.2.2 MEASURES OF PRINTABILITY

A clear understanding of the measures used to quantify printability is required in order to investigate the factors which influence printability. In this chapter, the different measures of extrudability, filament type, shape fidelity, and printing accuracy are considered. Table 6.1 lists the representative measurements for printability.

Table 6.1 Representative printability measurements.

Type	Measurement	Limitation/ condition	References
Extrudability			
Binary classification	Bioinks “unprintable” if flow could not be achieved at a maximum acceptable pressure	Nozzle detachment	Roehm and Madihally (2017)
	Bioinks “unextrudable” if flow could not be achieved at a maximum acceptable pressure	380 kPa	Zhu et al. (2016)
	Bioinks “unprintable” if a flowrate of 0.3 mL/h could not be achieved by their system	Unspecified	Wilson, Cross, Peak, and Gaharwar (2017)
Pressure under given conditions	Pressure generated by plunger displacement at a constant speed	0.2 mm/s plunger speed	Chung et al. (2013)
	Pressure required to achieve a given amount of material deposition per construct	100 mg construct	Gao, Gillispie, et al. (2018)
	Pressure required to achieve a minimum acceptable flowrate (lower bound) and pressure required to achieve a maximum acceptable flowrate (upper bound)	Varied with nozzle size	Paxton et al. (2017)
	Minimum pressure required to achieve consistent flow	Unspecified	Giuseppe et al. (2018)
Theoretical shear stress	Proportion of nozzle diameter to extrusion pressure	n/a	Webb and Doyle (2017)
Filament classification			
Submerged	Swelling, equivalent diameter, stretched, rough surface, overdeposited, compressed, and discontinuous		Jin, Chai, and Huang (2017)
Filament drop test	Droplet, filament		Schuurman et al. (2013)
	Droplet, smooth filament, and overgelled filament		Ouyang, Yao, Zhao, and Sun (2016)
Qualitative deposition	Straight and curvy		Habib, Sathish, Mallik, and Khoda (2018)
	Regular and irregular		Zhu et al. (2016) and Wilson et al. (2017)
Quantitative deposition	Uniformity ratio (filament perimeter normalized by length)		Gao, Gillispie, et al. (2018)
	Pr (pore perimeter normalized by pore area)		Ouyang et al. (2016)
	Standard deviation of filament heights and widths		Gohl et al. (2018)

Table 6.1 Representative printability measurements. *Continued*

Type	Measurement	Limitation/ condition	References
Shape fidelity			
Qualitative	Cross-hatch and anatomical shapes		Jin et al. (2017), Habib et al. (2018), Blaeser et al. (2013), Zhao, Li, Mao, Sun, and Yao (2015), Li et al. (2018), Lee, Kim, Seo, Park, and Cho (2017), Mouser et al. (2016), Schacht et al. (2015), Muller, Becher, Schnabelrauch, and Zenobi-Wong (2015), Muller, Ozturk, Arlov, Gatenholm, and Zenobi-Wong (2017), Abbadessa et al. (2016), Jiang, Xue, Liu, Han, and Wu (2017), Markstedt, Escalante, Toriz, and Gatenholm (2017), Ahlfeld et al. (2017), Bendtsen, Quinnett, and Wei (2017), Wang, He, and Zhang (2013), Laronda et al. (2017), Cohen et al. (2011), Tabriz, Hermida, Leslie, and Shu (2015), Suntornnond, Tan, An, and Chua (2017), Ahn et al. (2017), He et al. (2016), and Wu, Lin, Wenger, Tam, and Tang (2018)
Filament spreading (single layer)	Filament width		Roehm and Madihally (2017), Wilson et al. (2017), Chung et al. (2013), Giuseppe et al. (2018), Gohl et al. (2018), Lee et al. (2017), Ahn et al. (2017), Tirella, Orsini, Vozzi, and Ahluwalia (2009), Ng, Yeong, and Naing (2016), Lee, Lum, Seow, Lim, and Tan (2016), Daly, Critchley, Rencsok, and Kelly (2016), Markstedt et al. (2015), Kang, Hockaday, and Butcher (2013), and Diamantides et al. (2017)
	Filament height		Wilson et al. (2017), Gohl et al. (2018), Ahn et al. (2017), and Kang et al. (2013)
	Spreading ratio (filament width divided by nozzle diameter)		Daly et al. (2016)
	Aspect ratio (filament height divided by width)		Rutz, Hyland, Jakus, Burghardt, and Shah (2015)
	Pr (pore perimeter normalized by pore area)		Ouyang et al. (2016)

(Continued)

Table 6.1 Representative printability measurements. *Continued*

Type	Measurement	Limitation/ condition	References
Height maintenance	Critical height (maximum achievable height)		Rutz et al. (2015)
	Height of cylindrical structure		Duarte Campos et al. (2015)
	Height of five-layer tubular structure		Gao, Gillispie, et al. (2018)
Filament collapse	Angle of deflection of unsupported filament		Ribeiro et al. (2017)
	Pore area below an unsupported filament		Habib et al. (2018)
Printing accuracy			
Filament dimensions	Observation of broken filaments		Roehm and Madihally (2017), Li et al. (2018), Wang et al. (2013), Ng et al. (2016), and Lee, Lum, et al. (2016)
	Filament width		Roehm and Madihally (2017), Zhu et al. (2016), Wilson et al. (2017), Giuseppe et al. (2018), Webb and Doyle (2017), Habib et al. (2018), Gohl et al. (2018), Lee et al. (2017), Ahn et al. (2017), He et al. (2016), Tirella et al. (2009), Kang et al. (2013), Melchels, Dhert, Hutmacher, and Malda (2014), and Athirasala et al. (2018)
	Filament height		Wilson et al. (2017), Gohl et al. (2018), Ahn et al. (2017), and Kang et al. (2013)
	Micro-CT		Dahal et al. (2018) and Klar et al. (2019)
Pore dimensions	Pore area		Giuseppe et al. (2018), Habib et al. (2018), He et al. (2016), Diamantides et al. (2017), Duan, Hockaday, Kang, and Butcher (2013), Lee, Lee, et al. (2016), Kopf, Campos, Blaeser, Sen, and Fischer (2016), Murphy, Skardal, and Atala (2013), Law et al. (2018), and Duan, Kapetanovic, Hockaday, and Butcher (2014)
Filament merging	Minimum distance required between filaments without merging		Wust, Godla, Muller, and Hofmann (2014)
	Overlap distance of a filament printed at an acute angle		He et al. (2016)
	Length of fused segment between adjacent filaments with increasing distance between filaments		Ribeiro et al. (2017)

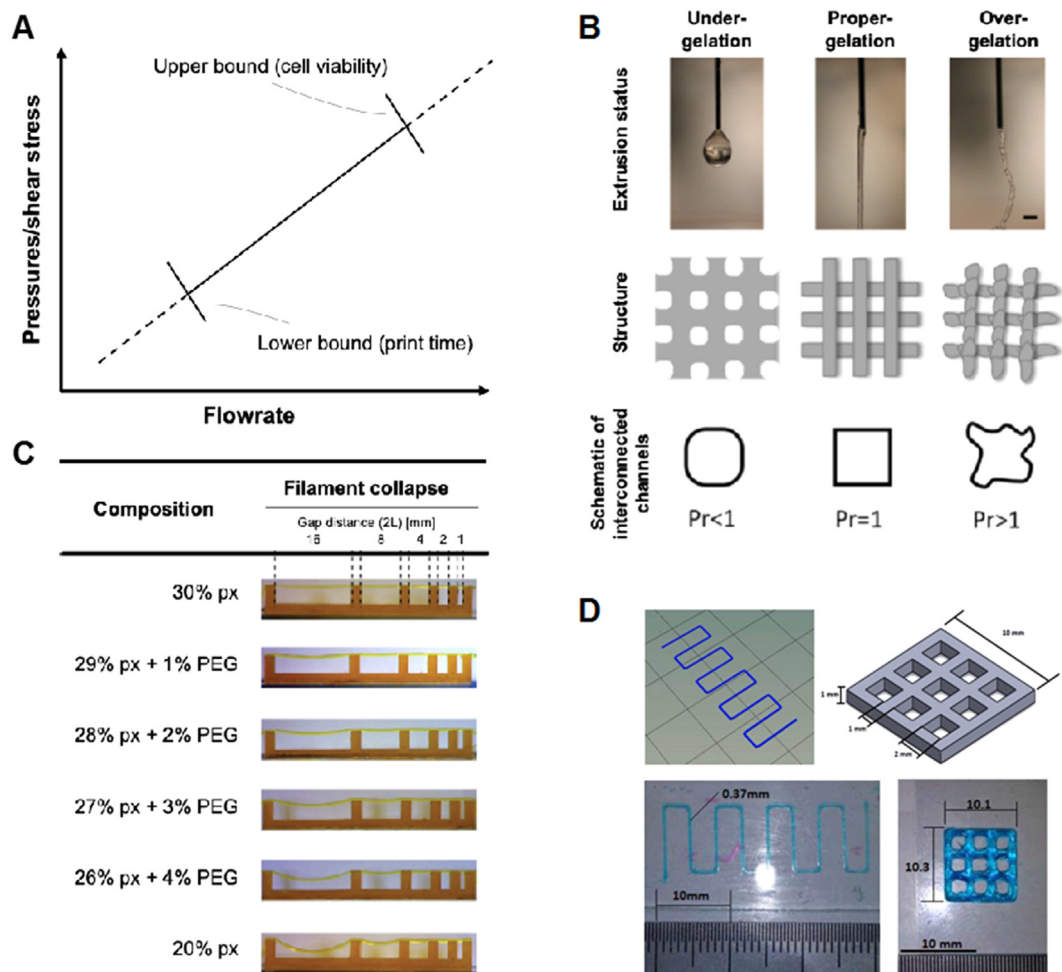
Table 6.1 Representative printability measurements. *Continued*

Type	Measurement	Limitation/ condition	References
Others			
Construct size	Weight of construct		Gao, Gillispie, et al. (2018)
Cell mixing	Whether cells could be mixed with a pipette		Mouser et al. (2016)
Homogeneity	Variability of extrusion force overtime		Chung et al. (2013), and Cohen et al. (2011)
<i>Reprinted with permission from Gillispie, Prim, et al. (2020).</i>			

Extrudability refers simply to how easily a bioink can be extruded through a small diameter nozzle, affecting total processing time and cell viability (Fig. 6.1A). Cell viability during the bioprinting process has been linked to the pressure experienced by the cells in the syringe, the shear stresses experienced by the cells in the nozzle, and the amount of time cells spend in the syringe (Aguado, Mulyasmita, Su, Lampe, & Heilshorn, 2012; Cheng et al., 2008; Habib et al., 2018; Lee & Yeong, 2015; Li et al., 2010; Ouyang et al., 2016; Roehm & Madihally, 2017; Yan, Nair, & Sun, 2010). Because of this, researchers sometimes indirectly quantify the extrudability of their system by live/dead assay immediately following the bioprinting process (Aguado et al., 2012; Cheng et al., 2008; Chung et al., 2013; Kang et al., 2013; Li et al., 2010; Li, Liu, & Lin, 2016; Li et al., 2018; Yan et al., 2010). Others have used extrusion pressure as the measure of extrudability. This can be either the pressure required to achieve a specified flowrate (Chung et al., 2013; Gao, Gillispie, et al., 2018; Giuseppe et al., 2018; Sweeney, Campbell, Hanson, Pantoya, & Christopher, 2017; Webb & Doyle, 2017), whether or not a given pressure was able to achieve flow (Roehm & Madihally, 2017; Rutz et al., 2015; Wilson et al., 2017; Zhu et al., 2016), or the minimum pressure which will cause flow (Smith, Basu, Saha, & Nelson, 2018; Sweeney et al., 2017) in their system.

Filament type can easily be determined qualitatively. Several researchers conduct this test by extruding the bioink into the air rather than on a substrate and classify the shape of the extruded material as either droplet or filament (Fig. 6.1B) (Habib et al., 2018; Ouyang et al., 2016; Paxton et al., 2017; Schuurman et al., 2013). Bioinks that form filaments can be further classified by whether that filament is smooth or uniform (Ouyang et al., 2016). Nonuniform filaments can be more easily identified after deposition as some other researchers have observed (Habib et al., 2018; Wilson et al., 2017; Zhu et al., 2016). However, the most robust measures of filament type are quantitative. After deposition, various dimensions can be made from the filaments including the perimeter of square pores relative to perfect squares (Ouyang et al., 2016), the perimeter of single filaments relative to a straight line (Gao, Gillispie, et al., 2018), and the variability of filament widths (Gohl et al., 2018).

The shape fidelity of a bioink is most frequently measured as the width and/or height of a single filament (Fig. 6.1C). While easy to evaluate, this measure is highly prone to influence by the quantity of material deposited and interactions with the surface substrate. Additionally, it does not capture the full multilayer, spatial behavior required of bioinks. Among measures which do, the *Pr* value developed by Ouyang et al. is an attractive measure. Although it only evaluates two printed layers, it also has the benefit of being able to detect nonuniform filaments and controlling for

**FIGURE 6.1**

Different aspects of printability. (A) Extrudability can be defined at any point along with the pressure–flowrate relationship. Notably, a minimum flowrate is required to achieve reasonable print times and maximum extrusion force is limited to achieve reasonable cell viability after extrusion. (B) Filament classification has been used to describe the types of filaments which a bioink can form. This example measure from Ouyang et al. shows how the phenomenon can both be observed qualitatively and measured quantitatively. (C) Shape fidelity refers to the ability of a bioink to maintain its structure upon deposition. The example here from Ribeiro et al. tests a bioink’s ability to form lateral pores. (D) Printing accuracy refers to the similarity of the printed structure to the original design as influenced by the printing conditions. This example from Giuseppe et al. uses zig-zag and cross-hatch structures to compare dimensions.

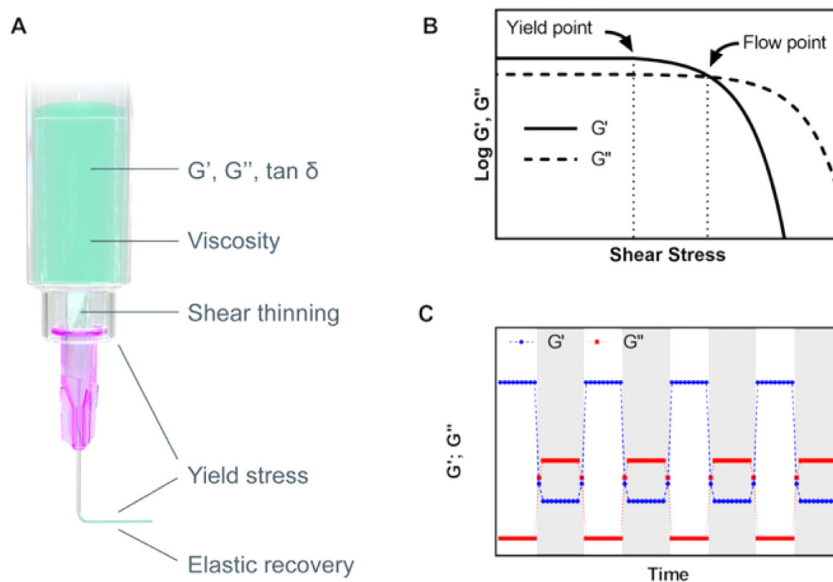
(B) Reprinted with permission from Ouyang et al. (2016); (C) Reprinted with permission from Ribeiro et al. (2017); (D) Reprinted with permission from Giuseppe et al. (2018).

material deposition by normalizing the perimeter to the area of the pore (Ouyang et al., 2016). Gao et al. quantified shape fidelity as the height of a five-layer tubular structure. Bioinks with poor shape fidelity sagged under the weight of multiple layers, resulting in a decreased height measurement (Gao, Gillispie, et al., 2018). Lastly, Ribeiro et al. measured the angle of the collapse of an unsupported filament printed between pillars of varying distances apart. Bioinks with poor shape fidelity have larger deflection angles as they cannot span the gaps as well (Ribeiro et al., 2017).

Printing accuracy has been measured in a variety of ways. Some of these measures are very similar to those for shape fidelity (Fig. 6.1D). The key distinction is that print accuracy measurements compare a common bioink under different printing conditions whereas shape fidelity measures compare different bioinks under common printing conditions. Most commonly, filament height, width, and pore size are used to assess printing accuracy. These are simple and important measures that can help ensure similarity between the designed structure and final structure, but they also test the accuracy of the printing system in the least strenuous fashion. Microcomputed tomography (μ CT) measurements have been used to compare entire constructs to the design computer-aided design. This provides a much more robust test but is difficult to conduct and requires expensive imaging equipment and software (Dahal et al., 2018; Klar et al., 2019). Weight/volume of the final construct may also be used to confirm the desired amount of material deposition (Gao, Gillispie, et al., 2018). Several researchers have also identified unintentional filament merging as an aspect of print accuracy. A particularly robust measure of this phenomenon involves printing a zig-zag pattern with the distance between lines increasing. The outcome measure is the “fused segment length” between two parallel filaments (Ribeiro et al., 2017). Ultimately, more measures of printing accuracy are needed to capture additional phenomena such as filament discontinuity, turn accuracy, errors in flow initiation and stoppage, and others.

6.3 RELATIONSHIPS BETWEEN PRINTING OUTCOMES AND RHEOLOGICAL PROPERTIES

Rheology is the branch of physics that studies the flow of matter. It has proven to be extremely useful for bioink development, and several rheological tests are applicable to bioprinting. Most hydrogels demonstrate non-Newtonian behavior (Jungst et al., 2016). In a Newtonian fluid, the shear rate-shear strain relationship is linear with viscosity remaining constant across strain rates. Hydrogels, on the other hand, are typically shear thinning, meaning their viscosity decreases as the shear rate increases (Oyen, 2014; Picout & Ross-Murphy, 2003). The result is beneficial for the purposes of flow. As the shear rate increases, the apparent viscosity of the material decreases, allowing flow to occur with lower pressure differentials and lower shear stresses on the material, and importantly cells, than would have occurred otherwise. This property is often measured via frequency sweeps, where the material is tested at a constant strain across a range of frequencies or shear rates (Fig. 6.2A) (Oyen, 2014; Picout & Ross-Murphy, 2003). Also important is the thixotropy of a material. Once a strain rate is applied, thixotropic materials will show a decrease in apparent viscosity over time. This change in viscosity in turn impacts the bioink’s flow with respect to time. Thixotropy is sometimes confused with shear thinning as both properties result in decreased viscosity, but shear thinning occurs with an increase in strain rate while thixotropy

**FIGURE 6.2**

Common rheological measures associated with bioink printability. (A) Behaviors of bioinks and commonly reported measures that play an impact on the printability of the bioink. (B) Shear-thinning behavior of bioinks (log scale) and demonstration of the yield point and flow point of the bioink. G' and G'' can be averaged from the linear viscoelastic region while yield stress can be defined at the crossover point between G' and G'' . (C) Recovery behavior of a bioink using different shear rates to model the extrusion phase.

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occurs over time at a given strain rate (Jungst et al., 2016). Materials may also be shear thickening (opposite of shear thinning) and rheopectic (opposite of thixotropic), but these are highly undesirable for bioprinting.

Hydrogel materials also demonstrate viscoelastic properties, meaning their behavior is determined both by viscous and elastic components. The elastic component is measured by the storage modulus, denoted by G' , and refers to energy that is stored in a material when deformation is applied. The viscous component is measured as the loss modulus, denoted G'' , and refers to energy that is lost when deformation is applied to the material (Oyen, 2014; Picout & Ross-Murphy, 2003). The loss tangent, or $\tan \delta$, is the ratio of loss modulus (G'') to storage modulus (G'). The $\tan \delta$ represents the relative contribution of viscous and elastic components to the material, with materials with values lower than “1” having a higher elastic contribution and materials with values higher than “1” having a higher viscous contribution. Typically, these properties are measured within the linear viscoelastic region (LVR) via strain or stress sweeps (Oyen, 2014; Picout & Ross-Murphy, 2003). Frequency is held constant, and either stress or strain is increased, with storage modulus, loss modulus, and $\tan \delta$ averaged throughout the LVR where they remain relatively constant (Fig. 6.2B).

Yield stress has been used to describe the initiation of flow and can be defined in multiple ways. Most commonly, a strain or stress sweep is used to gradually increase the stress on the material. As the material begins to flow, the storage modulus will drop dramatically. If dramatic and immediate enough, the stress at this drop-off can be defined as the yield stress (Nadgorny, Xiao, & Connal, 2017). For bioinks where this decrease is more gradual, the stress at which the storage modulus intersects with the loss modulus (i.e., where the $\tan \delta$ is >1 and the viscous properties begin to dominate the material's behavior) is considered the yield stress (Fig. 6.2B) (Kiyotake, Douglas, Thomas, Nimmo, & Detamore, 2019; Peak, Stein, Gold, & Gaharwar, 2018; Ribeiro et al., 2017; Shin et al., 2017; Wilson et al., 2017). Others have looked at both static and dynamic yield stress, defining static yield stress as the minimum required to initiate flow and dynamic yield stress as the minimum required to maintain flow (Smith et al., 2018). While most researchers stick to this concept during bioink development, the measurement and application of yield stress as a rheological measure is less straightforward than these measures may imply (Coussot, 2014; Moller, Fall, & Bonn, 2009).

Finally, rheology has been used to quantify the ability of bioinks to recover from the shear forces experienced during extrusion and return to the original material properties. Recovery tests are conducted in three steps. Initially, a very low shear rate is applied to the material, modeling pre-extrusion conditions. This is followed by a high shear rate intended to model extrusion. Finally, the material is returned to the initial shear rate, modeling postextrusion conditions (Fig. 6.2C). Recovery is typically defined as the end viscosity represented as a percentage of the initial viscosity. Recovery increases over time after the high shear rate is removed, so recovery must be expressed at a certain time (i.e., 10 and 60 seconds) after the high shear rate has been removed. Compared with other measures, recovery tests model the bioprinting process most closely. However, there is also the least amount of standardization between methods. Recovery can be measured by time to recovery (Peak et al., 2018) or percentage recovered (Dávila & d'Ávila, 2018; Kiyotake et al., 2019; Wilson et al., 2017). The recovered parameter can be viscosity (Dávila & d'Ávila, 2018; Peak et al., 2018) or G' (Kiyotake et al., 2019; Wilson et al., 2017). This can be measured across single (Dávila & d'Ávila, 2018; Kiyotake et al., 2019; Peak et al., 2018) or multiple (Wilson et al., 2017) cycles and the extrusion phase can be modeled via shear rate (Dávila & d'Ávila, 2018; Peak et al., 2018), strain (Wilson et al., 2017), or stress (Kiyotake et al., 2019).

6.3.1 EXTRUDABILITY

Among different aspects of printability, the relationships between rheological properties and extrudability are the most well understood. Direct measures of extrudability, such as experimentally derived pressure–flowrate curves, are rarely conducted except to validate a rheological model. As such, most researchers typically present rheological characterizations as a proxy for extrudability. The main rheological measure of extrudability is the viscosity, with higher viscosity resulting in lower extrudability. Due to the non-Newtonian behavior of bioinks, viscosity must be measured across a wide range of shear rates with the upper end preferably being in the range of that experienced by the bioink during extrusion. Many factors can influence a bioink's viscosity such as temperature, time, solvent, preparation methods, and many more. Hydrogel composition and concentration have been examined most frequently. The addition of material has been demonstrated to result in higher viscosity and, therefore, lower extrudability in many bioinks, including chitosan,

gelatin, alginate, Pluronic F127, κ -carrageenan (κ -CA), laponite, poly(2-hydroxyethyl methacrylate) (PHEMA), methylcellulose, xanthan, and others (Ahlfeld et al., 2017; Cheng et al., 2008; Li et al., 2016, 2018; Nadgorny et al., 2017; Paxton et al., 2017; Wilson et al., 2017; Wu et al., 2018).

Plotting several bioinks on the same viscosity vs shear rate graph can be sufficient for comparison within a single experiment. However, for cross-study comparisons and further modeling, it is helpful to model this behavior using the power-law relationship. By rheological testing a bioink over a range of shear rates ($\dot{\gamma}$) the resulting data can be fitted to a power-law equation,

$$\tau = K\dot{\gamma}^n$$

$$\tau = \eta\dot{\gamma}$$

$$\eta = K\dot{\gamma}^{n-1}$$

where τ is the shear stress and η is the viscosity as measured by the rheometer, K is the consistency index, and n is the flow index or power-law index. These two shear-thinning constants are very useful in modeling flow behaviors. K is related to the bioink's viscosity and is sometimes referred to as the “apparent” or “zero shear” viscosity (Lee & Yeong, 2015; Sarker & Chen, 2017). The degree to which a bioink is shear thinning can be inferred by the value of n , with values closer to “0” demonstrating more shear-thinning and values closer to “1” demonstrating more Newtonian behavior. Both of these values can be useful in comparing the extrudability of bioinks (Cheng et al., 2008; Kraut, Yenchesky, Prieto, Tovar, & Southan, 2017; Lee & Yeong, 2015; Li et al., 2016; Paxton et al., 2017; Sarker & Chen, 2017; Sweeney et al., 2017). For example, Nadgorny et al. developed a novel benzaldehyde-functionalized PHEMA with ethylenediamine (EDA) bioink and used these properties as proxies for extrudability to help optimize for the concentrations of PHEMA and EDA (Nadgorny et al., 2017).

Using these constants and the nozzle dimensions, the bioprinting system can be modeled in even further detail via the Hagen–Poiseuille equation. The pressure–flowrate relationship can be derived to predict the flowrate at a given pressure or pressure required to achieve a given flowrate (Dávila & d'Ávila, 2018; Duty et al., 2018; Khalil & Sun, 2007; Lee & Yeong, 2015; Li et al., 2016; Sweeney et al., 2017). Dávila et al. have applied this concept to the development of an alginate–laponite bioink with a highly desirable viscosity profile (Dávila & d'Ávila, 2018). Additionally, the shear rate and shear stress profiles can be derived to determine the forces cells are experiencing along the radius of the nozzle (Dávila & d'Ávila, 2018; Kang et al., 2013; Kraut et al., 2017; Li et al., 2016; Paxton et al., 2017). One of the limitations of these models is that they typically are not accurate for very low pressures. In a real-world system, a certain pressure must be overcome in order for the flow to occur, but these models will indicate flow even at low pressures. By incorporating the yield stress as the determining factor for the initiation of flow, Herschel–Bulkley models can be used to model flow behavior (Sarker & Chen, 2017; Smith et al., 2018). For example, Smith et al. examined Pluronic bioinks with various additives to optimize for chemical and flow characteristics (Smith et al., 2018). Furthermore, a no wall slip condition is commonly assumed when modeling bioink extrusion with these models. However, there is some evidence that neglecting wall slip will slightly underestimate the flowrate and more complex models that account for this boundary condition are more accurate (Lee & Yeong, 2015; Sarker & Chen, 2017). The influence of rheological properties on printing flowrate is well-characterized, but other printing conditions and parameters can still result in poor printing outcomes. Several researchers

have additionally used the rheological properties of their bioink to predict its deposition based on the flowrate and print speed (i.e., speed ratio) (Khalil & Sun, 2007; Lee & Yeong, 2015; Sarker & Chen, 2017).

The influence on cell damage is slightly less understood, although several factors have been identified. Using these models of extrusion, the shear stress experienced by the cells can be estimated, which has been directly related to their viability. Of particular note, shear stresses are highest near the wall of the nozzle, and viability is subsequently lowest in these regions (Cheng et al., 2008; Li et al., 2010; Yan et al., 2010). Flowrate and nozzle length have been used to estimate the extrusion duration of cells in the nozzle, which was found to decrease the viability of cells (Cheng et al., 2008). Other factors that have been implicated include waiting time in the syringe (Li et al., 2010) and extensional flow at the syringe to nozzle transition (Aguado et al., 2012). Others have found no difference in cell viability at different extrusion pressures or shear stresses (Kang et al., 2013). Complicating the matter further, while the direction of these relationships seems likely to hold regardless, the cell types and hydrogel carriers used may influence the magnitude of these effects. Further research is needed into the different causes of cell damage during the bioprinting process, especially in relation to the rheological properties of the bioink cell carrier. Such studies would aid significantly in future bioink development.

6.3.2 FILAMENT CLASSIFICATION

The rheological influence on filament classification is still poorly understood and has mainly remained uncharacterized. Still, some trends can be observed when viewing the body of literature. Schuurman et al. showed that at 37°C their gelatin methacrylate (GelMA)-only bioink formed droplets even at concentrations up to 20% (w/v). The addition of 2.4% (w/v) hyaluronic acid resulted in smooth filaments and improved printing outcomes (Schuurman et al., 2013). Paxton et al. classified the filaments of different bioinks (Paxton et al., 2017). They looked at different concentrations of Pluronic F127, different concentrations of alginate, and different degrees of alginate crosslinking. No rheological characterizations were done on bioinks that formed droplets because they were deemed unprintable. However, both Pluronic and alginate transitioned from droplet to smooth filament formation with increasing concentration. Alginate was also able to undergo this transition by increasing its degree of crosslinking via CaCl_2 concentration. Dávila et al. added laponite to their 1% alginate bioink and saw a transition from droplet to smooth filament between 4% and 5% laponite, corresponding with increases in viscosity, G' , and G'' (Dávila & d'Ávila, 2018). Habib et al. mixed 4% alginate with varying carboxymethylcellulose (CMC) concentrations and found a transition from droplet to smooth filament formation between 1% and 2% CMC (Habib et al., 2018). Ouyang et al. showed the effect of concentration, temperature, and holding time on gelatin–alginate bioinks (Ouyang et al., 2016). In their work, droplet formation at a given temperature was seen in bioinks which did not gel (defined as a crossover between G' and G'') at that temperature.

Ouyang et al. also found the formation of nonuniform filaments to occur with higher gelatin concentrations, lower temperatures, and longer holding times. As each of those factors increased gelation, the authors attributed the nonuniform filaments to “over-gelation” (Ouyang et al., 2016). Gao et al. related rheological measures to filament type directly (Gao, Gillispie, et al., 2018). Using varying concentrations of gelatin and alginate, the researchers showed a transition from smooth to

nonuniform filaments as the loss tangent decreased. The transition occurred approximately between a loss tangent of 0.25 and 0.45. However, this window was not found to be predictive when applied to other bioinks. Kiyotake et al. showed their pentanoate-functionalized hyaluronic acid hydrogel-based bioinks to increase in viscosity and G' , decrease in recovery percentage, and have higher yield stress with increasing concentration (Kiyotake et al., 2019). At the highest concentrations, the bioinks exhibited nonuniform filaments. They attributed this loss of uniformity to a dramatic increase in yield stress (> 1000 Pa) and poor recovery from a high shear rate ($< 85\%$). Conversely, Zhu et al. looked at polyion complex (PIC) hydrogels and found lower hydrogel concentrations to result in both decreased viscosity and nonuniform filaments (Zhu et al., 2016).

While it is inconclusive from the current studies how rheological properties govern the droplet to uniform filament transition, some notable deductions can be made. Transitions from droplet to smooth filament have been found by increasing hydrogel concentration, ionic crosslinking, and thermal gelation. In general, this transition represents a change in the factor which dominates the bioink behavior. At droplet formation, bioink behavior is dominated by surface tension and water molecule interactions. For filament formation, the polymer network begins to dominate behavior as hydrogel concentration and gelation increase. However, further increases in hydrogel concentration and/or gelation beyond smooth filament formation are typically required to accomplish good shape fidelity (Gao, Gillispie, et al., 2018; Habib et al., 2018; Ouyang et al., 2016). All bioinks which form droplets will have poor printability, but not all bioinks which form smooth filaments will have good printability. Therefore droplet formation is better viewed as a prescreening tool and the bare minimum barrier used to quickly eliminate bioink candidates.

The underlying cause of the transition from smooth to nonuniform filaments is even less clear. The contradictory results between gelatin-based and PIC-based bioinks likely mean different mechanisms are at play in the cause of these phenomena between the two hydrogels. Furthermore, some hydrogels (such as Pluronic) do not seem to exhibit this behavior regardless of hydrogel concentration or degree of crosslinking. As a result, further research into this phenomenon is needed before any major conclusions can be drawn.

6.3.3 SHAPE FIDELITY

It is also difficult to determine which rheological measurements influence shape fidelity. Very few studies have attempted to directly define this relationship. However, many studies have looked at both rheology and shape fidelity. Most commonly, this is done while optimizing for hydrogel concentration within a bioink (Ahlfeld et al., 2017; Chung et al., 2013; Dávila & d'Ávila, 2018; Habib et al., 2018; Jessop et al., 2019; Kiyotake et al., 2019; Li et al., 2016, 2018; Nadgorny et al., 2017; Peak et al., 2018; Rutz et al., 2015; Shin et al., 2017; Wilson et al., 2017). Hydrogel concentration influences many rheological parameters simultaneously, which is a major obstacle in studying the rheology–shape fidelity relationship. The most frequently rheological measure examined is viscosity. As hydrogel concentration increases, so does the viscosity for most materials. In these studies, a subsequent increase in shape fidelity is also typically seen (Ahlfeld et al., 2017; Chung et al., 2013; Habib et al., 2018; Jessop et al., 2019; Kiyotake et al., 2019; Li et al., 2016, 2018; Nadgorny et al., 2017; Peak et al., 2018; Shin et al., 2017). However, viscosity encompasses only a single portion of bioink behavior, namely, its resistance to flow. The viscoelastic properties of a bioink have also been shown to play an important role. A bioink's complex modulus (G^*) also typically

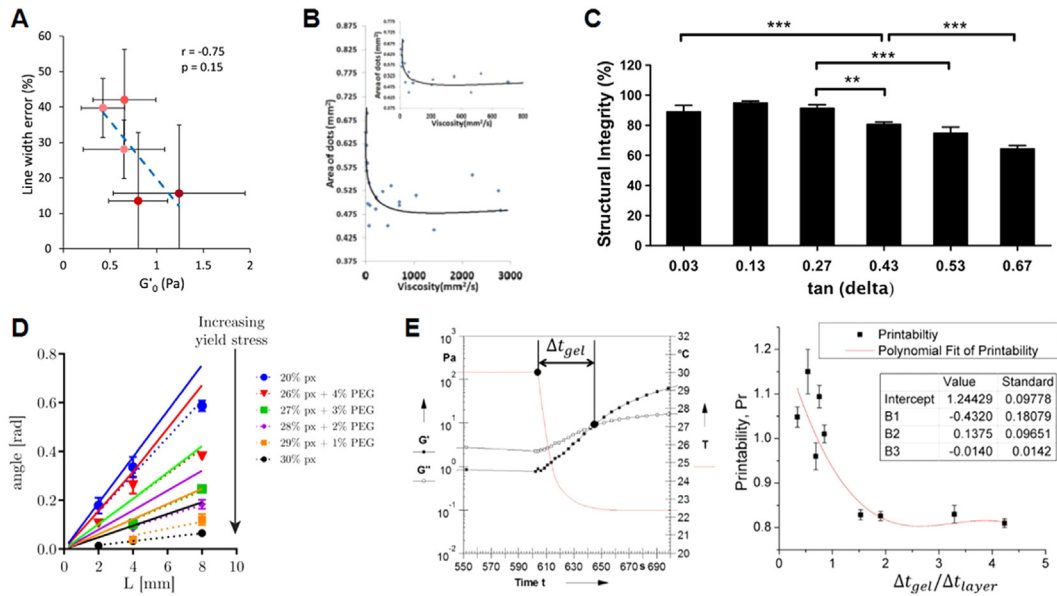
increases with concentration. This increase may be due to an increase in G' , G'' , or, most commonly, both (Dávila & d'Ávila, 2018; Jessop et al., 2019; Nadgorny et al., 2017; Peak et al., 2018; Rutz et al., 2015; Shin et al., 2017). Even as both G' and G'' increase, one may come to dominate the behavior as the relative contributions (quantified as the loss tangent) change (Gao, Gillispie, et al., 2018). G^* , G' , and G'' have all been linked to shape fidelity indirectly through bioink concentration. As the measure of elasticity, G' has received the most attention among them (Dávila & d'Ávila, 2018; Jessop et al., 2019; Nadgorny et al., 2017; Peak et al., 2018; Rutz et al., 2015; Shin et al., 2017).

The yielding behavior of a bioink is also frequently studied alongside changes in shape fidelity. The concept here is that as bioink's yield strength increases, it can resist greater forces before plastically deforming. As hydrogel concentration increases, so does the amount of stress required to initiate flow. This increase has been linked to shape fidelity in several studies (Kiyotake et al., 2019; Nadgorny et al., 2017; Peak et al., 2018; Rutz et al., 2015; Shin et al., 2017). Rutz et al. additionally reported on the strain at the yield point, which decreased with increasing concentration and shape fidelity, and G' at the yield point, which increased with hydrogel concentration and shape fidelity (Rutz et al., 2015). Additionally, this relationship does not necessarily hold when adding a new hydrogel to the bioink, as Wilson et al. found the addition of laponite to their κ -CA bioink to dramatically decrease the yield stress while simultaneously improving its shape fidelity (Wilson et al., 2017).

Lastly, the recovery behavior of a bioink has also been linked indirectly to shape fidelity via hydrogel concentration. Wilson et al. attributed the improved fidelity of their laponite supplemented bioink (despite its decrease in yield stress and G') to its enhanced recovery behavior (93%–99% recovery of initial G' vs 69% for the κ CA only bioinks) (Wilson et al., 2017). Also looking at laponite, Peak et al. found a decrease in recovery time corresponded to an improved shape fidelity for their PEG-based bioinks (Peak et al., 2018). Conversely, Kiyotake et al. saw a decrease in recovery percentage with increasing shape fidelity for a pentanoate-functionalized HA-based bioink (Kiyotake et al., 2019).

Several studies have attempted to directly relate rheological measures to shape fidelity. Diamantides et al. compared several rheological measures to the shape fidelity of their collagen-based bioinks, including G' pre- and post-UV crosslinking, crosslinking rate, and time required to crosslink. They found that, under their range of testing conditions, G' prior to crosslinking was the best predictor of shape fidelity (Fig. 6.3A) (Diamantides et al., 2017). Jia et al. related the shape fidelity of alginate hydrogels to their viscosity. By varying the alginate concentration and oxidation percentage, they found that higher viscosities resulted in better shape fidelity (Fig. 6.3B) (Jia et al., 2014). Smith et al. investigated four different additives at two concentrations each by incorporating them into their methacrylated Pluronic bioink. Shape fidelity was assessed by the filament diameter upon deposition relative to that of the nozzle diameter. The authors related this measure to their bioink's dynamic yield stress, with high yield stress correlating to high shape fidelity. However, the researchers did not control for flowrate and it is unclear whether the changes in filament diameter were a result of decreased shape fidelity or simply different volumes of bioink being deposited (Smith et al., 2018).

Results from Gao et al. directly relate loss tangent to shape fidelity by measuring the height of a 5-layer tubular structure. The height of the structure was inversely related to the loss tangent (G'/G'') and loss modulus (G'') of their gelatin–alginate bioinks (Fig. 6.3C) (Gao, Gillispie, et al., 2018).

**FIGURE 6.3**

Identified relationships between rheology and shape fidelity. (A) Diamantides et al. related the storage modulus of their bioinks to filament width relative to nozzle size. (B) Jia et al. related the viscosity of their bioinks to the area covered by printed dots. (C) Gao et al. related the $\tan \delta$ (loss tangent) of their bioinks to the height of a 5-layer tubular structure. (D) Ribeiro et al. related the yield stress of their bioinks to its angle of deflection across unsupported gaps of varying distances. (E) Ouyang et al. related the gelation kinetics of their bioinks to the shape of horizontal pores, quantified using their Pr value.

(A) Reprinted with permission from Diamantides et al. (2017); (B) Reprinted with permission from Jia et al. (2014); (C) Reprinted with permission from Gao, Gillispie, et al. (2018); (D) Reprinted with permission from Ribeiro et al. (2017); (E) Reprinted with permission from Ouyang et al. (2016).

Ribeiro et al. developed a theoretical model for their measure of shape fidelity. They printed Pluronic-PEG bioinks across pillars of varying distances apart, leaving the bioink unsupported between pillars. The following equation was derived to predict the angle of deflection (θ) of the unsupported filament:

$$\theta = \sin^{-1} \left(\frac{\rho g L}{\sigma_{\text{yield}}} \right)$$

where p is the bioink density, g is the gravitational acceleration, L is half the length between pillars, and σ_{yield} is the yield stress of the bioink as measured on a rheometer. Their results showed a strong negative correlation between yield stress and deflection angle. The model slightly overestimated the angle of deflection but showed similar trends as the experimental data across different bioinks and gap distances (Fig. 6.3D) (Ribeiro et al., 2017). Ouyang et al. showed, using thermo-sensitive alginate–gelatin bioinks, that gelation time impacted printability. Bioinks which did not

gel as quickly did not maintain their shape as well, especially if the gelation time was longer than the time it took to print each layer (Fig. 6.3E) (Ouyang et al., 2016).

To summarize, bioink shape fidelity has been positively correlated to hydrogel concentration, thermal crosslinking, viscosity, G' , yield stress, and recovery abilities and negatively correlated with G'' and loss tangent. A major limitation in studying this relationship is the difficulty of isolating individual parameters. It seems likely at this point in time that each of these factors may play a role in bioink shape fidelity. More complex models of bioink behavior, such as via finite element analysis (Gohl et al., 2018), may be needed to predict printing outcomes.

6.3.4 IMPACT OF CELL DENSITY ON PRINTING OUTCOMES

The effect of cell density on printing outcomes has been examined (Diamantides, Dugopolski, Blahut, Kennedy, & Bonassar, 2019; Gillispie, Han, et al., 2020). Most studies looking at the influence of cell density on hydrogel bioink behavior have only examined density as high as 10×10^6 cells/mL and did not investigate the printability directly, examining rheological properties instead (Billiet, Gevaert, De Schryver, Cornelissen, & Dubruel, 2014; Cheng et al., 2008; Maisonneuve, Roux, Thorn, & Cooper-White, 2013). One study tested a density up to 100×10^6 cells/mL with filament width determination as a printing outcome (Diamantides et al., 2019). The inclusion of cells in a collagen-based bioink reduced the line width at cell densities as low as 5×10^6 cells/mL. In that study, collagen was printed in the solution phase onto a 37°C plate which then induced gelation. The addition of cells likely increased the viscosity of collagen prior to gelation, reducing the amount of spread upon deposition.

Another study investigated variations between rheological properties of cell-seeded bioinks compared with acellular bioinks (Gillispie, Han, et al., 2020). This study found statistically significant increases in storage and loss modulus when seeding bioinks with 20×10^6 /mL and 40×10^6 cells/mL. It is also observed slight changes in yield stress which increased at a density of 5×10^6 /mL but decreased at higher cell densities. Overall, the shear-thinning ability of the bioinks remained the same. Although these bioinks exhibited statistically significant differences in rheological properties, the results showed that there were no statistical differences in the printing outcomes such as radial accuracy, wall thickness, height of structures, nor Pr value when printing tubular and cross-hatch pattern (Gillispie, Han, et al., 2020). This emphasizes the current challenges with correlating rheological properties to printing outcomes.

6.4 RELATIONSHIPS BETWEEN PRINTING OUTCOMES AND PROCESS PARAMETERS

Some measures of printability are influenced by the printing process. These measures of printability include: line width, corner resolution, corner regularity, pore size, and filament horizontal holding (Diamantides et al., 2017; Paxton et al., 2017; Suntornnond, Tan, An, & Chua, 2016). Measures of printability are controlled by varying sets of process parameters. These measures of printability have been assessed visually, either qualitatively or quantitatively. Qualitative assessments have been performed by eye and judgment (Habib et al., 2018; He et al., 2016). Quantitative assessments

involve a photograph from either a camera (Giuseppe et al., 2018; Habib & Khoda, 2018; Habib et al., 2018; Vijayavenkataraman, Vialli, Fuh, & Feng Lu, 2019) or microscope (Ahn et al., 2017; Habib & Khoda, 2018; Habib et al., 2018) followed by image analysis with software such as ImageJ (Ahn et al., 2017; Giuseppe et al., 2018; Gohl et al., 2018; Habib & Khoda, 2018; Habib et al., 2018; Webb & Doyle, 2017).

6.4.1 PROCESS PARAMETERS

The most examined measure of printability is the line width. Line width increases with increasing nozzle diameter, extrusion pressure, and nozzle height. Line width also increases with decreasing feedrate and line pitch. These relationships have been demonstrated over limited independent variable ranges and result in limited dependent variable ranges (Table 6.2). Some independent variable ranges overlap. Differences can be attributed to differences in materials (as different hydrogels were tested in each study), which vary in rheological and material properties, and by differences in other process parameters when isolating correlation among parameters of interest.

Line height can be controlled by extrusion pressure, feedrate, nozzle height, or recovery time. The only report of an extrusion pressure influence on the line height employed an overdeposition methodology, whereby the flowrate resulted in an equal or excessive filament height as compared to the layer height. These researchers reported line heights of 1–9 times the layer height (Ahn et al., 2017). Conversely, most printing methodologies constrain the flowrate such that the resulting filament deposition is equal to the layer height. Extrusion pressure, flowrate, and feedrate are freely adjusted within a range that results in filaments equal to the layer height. If extrusion pressure, flowrate, and feedrate yield ideal within-layer printability, but the between-layer printability is unacceptable, nozzle height is then adjusted to match. At no time can the resulting filament height be continuously less than the layer height, as this will result in a cumulative increase in nozzle height and print failure. It may be advantageous for the resulting deposition height to be slightly greater than the nozzle position so as to create interference and better adhesion between layers.

When hydrogel filaments of different orientations overlap (often reported at 90° angles), filaments merge at the point of contact, and surface tension can draw out sharp contact angles (Habib et al., 2018; He et al., 2016). The degree of filament diffusion has been correlated with line pitch, in which filament diffusion increases with decreasing pitch. Large line pitch minimizes filament diffusion, but there will always be some small amount which occurs. If pores are too small, filament diffusion can be so severe that the initial pore disappears. Similarly, Ribeiro et al. have developed a filament fusion test that shows how changes in the distance between filaments affect the fusion between filaments. As the line pitch of their cross-hatch design increased, the length of the fused section between filaments decreased (Ribeiro et al., 2017).

As a process parameter, line pitch is user-controlled and determined prior to printing. Therefore the influence of line pitch on printability is necessary to understand. Line pitch has been shown to influence the filaments deposited on subsequent layers, with greater distances between printed filaments on the supporting layer resulting in higher filament collapse on the supporting layer (Habib & Khoda, 2018; Habib et al., 2018; Ribeiro et al., 2017; Therriault, White, & Lewis, 2007). For a given material, some increased line pitch results in 100% filament collapse and all greater line pitches have complete collapse. For example, a line pitch of 2 mm was achieved with alginate before the complete collapse, and the addition of CMC achieved 3 mm at a low concentration and

Table 6.2 Influence of various printing parameters on printing outcomes.

	Process parameter	Process parameter values	Range of resulting printability measure	Direction ^a	Materials
Line width	Nozzle diameter	210 μm (27 G), 260 μm (25 G), 514 μm (21 G) (Suntornnond et al., 2016)	220.72–3215.85 μm	↑	24.5% Pluronic F127
		30 G, 27 G, 25 G (Webb & Doyle, 2017)	270–9170 μm	↑	7% alginate + 8% gelatin
		100 μm (32 G), 150 μm (30 G), 230 μm (27 G), 300 μm (25 G), 400 μm (23 G) (Giuseppe et al., 2018)	170–570 μm	↑	7% alginate + 8% gelatin
	Extrusion pressure	100, 200, 300 kPa (Suntornnond et al., 2016)	220.72–3215.85 μm	↑	24.5% Pluronic F127
		100, 150, 200, 250 kPa (Webb & Doyle, 2017)	270–9170 μm	↑	7% alginate + 8% gelatin
		4, 41, 55, 69, 83, 103 kPa (5, 6, 8, 10, 12, 15 psi) (Habib et al., 2018)	~ 450 to ~ 1750 μm	↑	4% alginate + CMC
		69, 83, 97, 110, 138 kPa (10, 12, 14, 16, 20 psi) (Habib & Khoda, 2018)	~ 480 to ~ 1260 μm	↑	4% alginate + CMC + montmorillonite
	Feedrate	40, 60, 80 kPa (Ahn et al., 2017)	~ 450 to ~ 4300 μm	↑	dECM (porcine skin)
		4 to 9 mm/s (He et al., 2016)	~ 550 to ~ 1130 μm	↓	2.5% alginate + 8% gelatin
		10, 20, 30 mm/s (Suntornnond et al., 2016)	220.72–3215.85 μm	↓	24.5% Pluronic F127
		1, 2, 3, 4, 5, 6 mm/s (Webb & Doyle, 2017)	270–9170 μm	↓	7% alginate + 8% gelatin
		4, 5, 6, 7, 8, 9, 10 mm/s (Habib et al., 2018)	~ 450 to ~ 1350 μm	↓	4% alginate + CMC
		0.8, 2.1, 3.3 mm/s (Ahn et al., 2017)	~ 450 to ~ 4300 μm	↓	dECM (porcine skin)
	Nozzle height	0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1 mm (He et al., 2016)	~ 960 to ~ 1270 μm	↑	2.5% alginate + 8% gelatin
		0.4, 0.7, 0.9, 1.1, 1.3, 1.5 mm (Habib et al., 2018)	~ 570 to ~ 1270 μm	↑	4% alginate + CMC

(Continued)

Table 6.2 Influence of various printing parameters on printing outcomes. *Continued*

	Process parameter	Process parameter values	Range of resulting printability measure	Direction ^a	Materials
Line height	Line pitch	300, 400, 500 μm (Gohl et al., 2018)	~ 420 to ~ 450 μm	-	4% CNF
		300, 400, 500 μm (Gohl et al., 2018)	~ 720 μm	-	Ink 6040
		1, 2, 3, 4, 5 mm (He et al., 2016)	~ 680 to ~ 860 μm	↓	2.5% alginate + 8% gelatin
		40, 60, 80 kPa (Ahn et al., 2017)	~ 250 to ~ 2250 μm	↑	dECM (porcine skin)
Pore size (1 – filament diffusion rate)	Extrusion pressure	40, 60, 80 kPa (Ahn et al., 2017)	~ 250 to ~ 2250 μm	↑	dECM (porcine skin)
		40, 60, 80 kPa (Ahn et al., 2017)	~ 250 to ~ 2250 μm	↑	dECM (porcine skin)
		40, 60, 80 kPa (Ahn et al., 2017)	~ 250 to ~ 2250 μm	↑	dECM (porcine skin)
		40, 60, 80 kPa (Ahn et al., 2017)	~ 250 to ~ 2250 μm	↑	dECM (porcine skin)
Filament collapse	Feedrate	5, 10, 20 mm/s (Gohl et al., 2018)	~ 280 to ~ 460 μm	↓	4% CNF
		5, 10, 20 mm/s (Gohl et al., 2018)	~ 280 to ~ 460 μm	↓	4% CNF
		5, 10, 20 mm/s (Gohl et al., 2018)	~ 280 to ~ 460 μm	↓	4% CNF
		5, 10, 20 mm/s (Gohl et al., 2018)	~ 280 to ~ 460 μm	↓	4% CNF
	Nozzle diameter	100 μm (32 G), 150 μm (30 G), 230 μm (27 G), 300 μm (25 G), 400 μm (23 G) (Giuseppe et al., 2018)	77%–99%	↓	7% alginate + 8% gelatin
		100 μm (32 G), 150 μm (30 G), 230 μm (27 G), 300 μm (25 G), 400 μm (23 G) (Giuseppe et al., 2018)	77%–99%	↓	7% alginate + 8% gelatin
		100 μm (32 G), 150 μm (30 G), 230 μm (27 G), 300 μm (25 G), 400 μm (23 G) (Giuseppe et al., 2018)	77%–99%	↓	7% alginate + 8% gelatin
		100 μm (32 G), 150 μm (30 G), 230 μm (27 G), 300 μm (25 G), 400 μm (23 G) (Giuseppe et al., 2018)	77%–99%	↓	7% alginate + 8% gelatin
Filament collapse	Line pitch	1, 2, 3, 4, 5 mm (He et al., 2016)	$\sim 80\%$ to $\sim 96\%$	↑	2.5% alginate + 8% gelatin
		1, 2, 3, 4, 5, 6 mm (Habib et al., 2018)	0% to $\sim 90\%$	↑	4% alginate + CMC
		1, 2, 3, 4, 5, 6 mm (Habib & Khoda, 2018)	0% to $\sim 95\%$	↑	4% alginate + CMC + montmorillonite
		1, 2, 3, 4, 5, 6 mm (Habib & Khoda, 2018)	0% to $\sim 95\%$	↑	4% alginate + CMC + montmorillonite
	Line pitch	2, 4, 6, 8, 10 mm (Vijayavenkataraman et al., 2019)	0%–93.75%	↑	collagen/PPy-b-PCL
		1, 2, 3, 4, 5, 6 mm (Habib et al., 2018)	0%–100%	↑	4% alginate + CMC
		1, 2, 3, 4, 5, 6 mm (Habib & Khoda, 2018)	0%–100%	↑	4% alginate + CMC
		2, 4, 8, 16 mm (Ribeiro et al., 2017)	0.02–0.58 rad	↑	Poloxamer + PEG

^aDirection of the relationship between printability and printing condition.
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at least 6 mm at higher concentrations (Habib et al., 2018). The addition of montmorillonite to alginate decreased the line pitch at which complete collapse occurred, from 3 mm down to 2 mm, which was likely due to the increased weight of the montmorillonite filament without an appreciable increase in yield strength (Habib & Khoda, 2018). The usage of the filament collapse test has suggested that varying degrees of filament sag are permissible, but so far, acceptable, and unacceptable ranges have not been identified or discussed.

Corner resolution describes the sharpness of a corner. Current measures of corner resolution are qualitative, being either sharp or curved/rounded. Nozzle height affects corner resolution: increasing nozzle height reduces corner resolution, shifting the deposition shape from sharp to curved (Habib et al., 2018; He et al., 2016). Corner regularity has been used to describe the degree of overdeposition at a corner. As the corner angle decreases below a right angle, extrudate increasing overlaps. For sharp corners of small angles measures, extrudate overlaps considerably and causes the corner to bulge. To prevent the buildup of material in sharp angle corners, the rate of material deposition and be decreased or feedrate can be increased (He et al., 2016).

6.4.2 IMPROVING PRINTABILITY BY PROCESS PARAMETERS

The main researched printability measures affected by process parameters include line width and line height (Fig. 6.4). This distinction is from quantitative-only assessments in the reporting literature; qualitative assessments were not included. However, it has been summarized their findings qualitatively since factors such as differing materials account for significant differences in results. Regardless of the difference in material properties as they play out in different extrusion printing systems, the trends are universal.

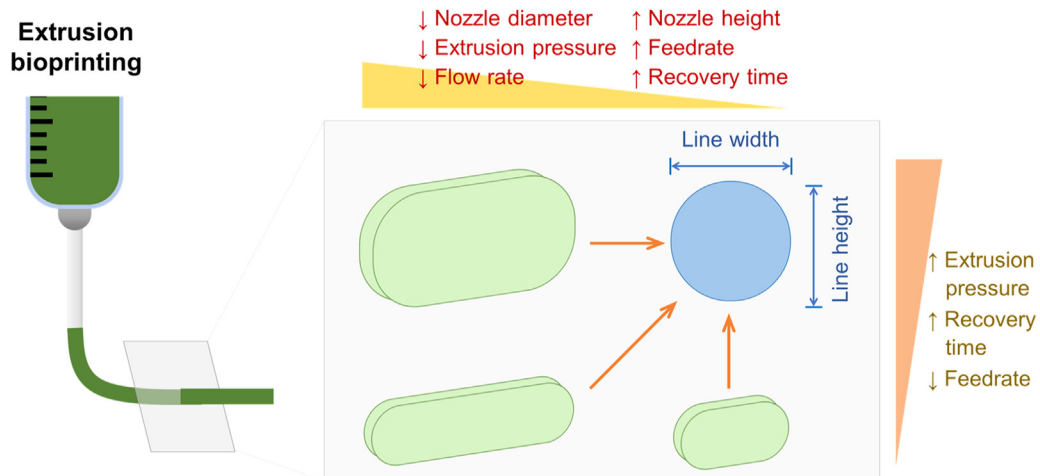


FIGURE 6.4

Relationships among printability measures and process parameters to control line width and line height (cross-sectional geometry).

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Process parameters have a role in printability. In general, accuracy is improved by reducing line width and increasing line height. Most materials and printing conditions result in filaments that are too wide and that sag over their cross-sectional area (reducing line height). The combined effect is a reduction in pore area (between printed lines). Changes that reduce line width and increase line height will increase pore area, improving printability. Some of the influential process parameters affect more than one printability measure. Recovery time affects both line width and line height. Increasing recovery time decreases line width and increases line height. Fortunately, in the case of recovery time, a single change in this one parameter will improve printability in two different measures (line width and line height). Extrusion pressure and feedrate, however, cause divergences in line width and line height. Increasing feedrate (or decreasing extrusion pressure) decreases line width (an improvement) but decreases line height (a detriment). There is only one report of recovery time affecting filament height as a measure of printability, and this report only investigated alginate and graphene oxide doped alginate (Li et al., 2016).

While some research has identified the connection between flowrate and line width, none has identified a connection between flowrate and line height. Since flowrate is directly proportional to extrusion pressure, the relationship between flowrate and line height can be similar to the one between extrusion pressure and line height. A future study should examine this relationship and use several different materials to show the scope of applicability.

6.5 MODELS FOR PRINTABILITY

Various computational models have been developed to both analyze and predict printing outcomes. One of the most popular *in silico* models is the power-law model which considers rheological data as well as printing parameters to predict shear-thinning coefficients. The model relies on inputs such as viscosity shear rate, needle length and radius, and extrusion pressure and velocity but is restricted to static and linear fluid flow with incompressible materials (Paxton et al., 2017; Schwab et al., 2020). The Herschel–Bulkley equation is a numerical model used for printability to predict flow behavior, flowrate, and yield stress. It considers the rheological properties of the bioink such as shear stress and shear rate and is applicable for non-Newtonian fluids. It can also handle nonlinear shear stress and strain behaviors as well as more complex material properties such as wall slipping which is the reduced viscosity near the needle wall (Sarker & Chen, 2017; Schwab et al., 2020). Computational modeling also promises to reduce some of the poor printing outcomes by relating printability to material properties. Lopez et al. developed a numerical simulation, called FingerMap, which aims to predict *in silico* the collapse of a voxelized 3D design. They validated this program with three silicone formulations with increasing yield stress and defined a printability domain according to overhang angle and the z-position of each voxel. They then printed two anatomical models and compared the theoretical results from the program and the experimental results from the models and observed a good correlation between the two (Lopez, Marquette, & Courtial, 2021).

Real-time optimization of process parameters from quantitative data has the potential to improve printing outcomes as demonstrated by Wang et al.'s use of an iterative feedback bioprinting approach which relies on optical coherence tomography (OCT) or through micro-CT imaging after the printing process (Fig. 6.5A). In this approach, a custom OCT system analyzes the

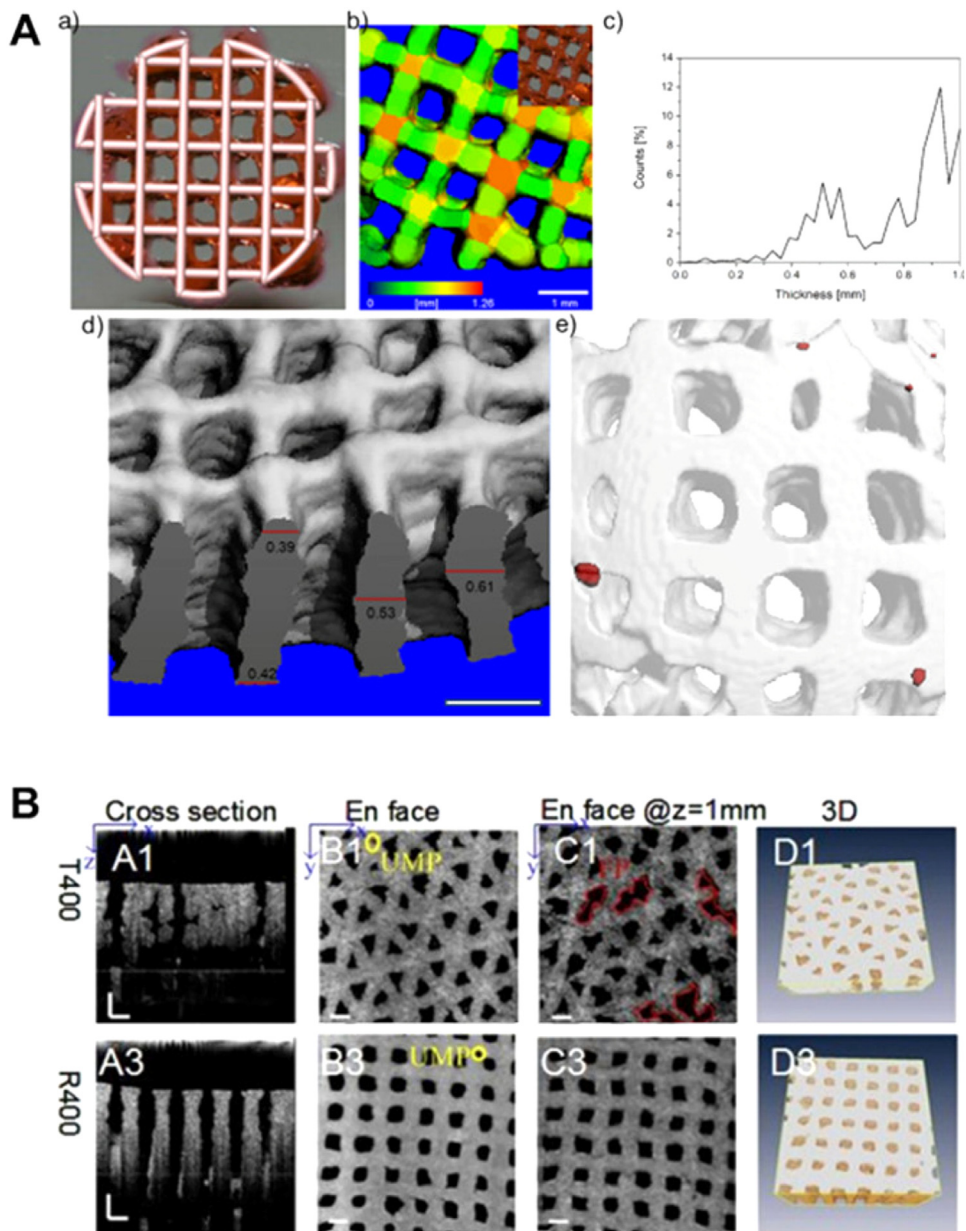


FIGURE 6.5

Micro-CT and optical coherence tomography (OCT) utilizing for the analysis of printing outcomes. (A) Use of micro-CT to assess shape fidelity of 3D-printed HA-based hydrogel: (a) optical image of a 3D-printed lattice grid overlapped with its 3D computer-aided design model; (b) micro-CT 3D reconstruction of the printed construct, where the color represents the thickness; (c) Strut thickness distribution in the 3D reconstruction; (d) micro-CT cross-section of a 3D-printed construct of multiple layers illustrating overlaying accuracy; (e) 3D reconstruction image showing air pockets in red. Scale bars 1 mm. (B) OCT imaging of gelatin/alginate hydrogel with different architectures illustrating (A1, A3) the cross-section; (B1, B3) surface; (C1, C3) hydrogel at 1 mm depth; and (D1, D3) 3D observation of hydrogel matrix. Scale bars 500 μ m. *UMP*, Undefined micropores.

(A) Reprinted with permission from (Petta, Grijpma, Alini, Eglin, & D'Este, 2018); (B) Reprinted with permission from (Wang, Zhang, Zhou, & Luo, 2016)

two-dimensional and 3D printing outcomes such as pore size, surface area, and pore volume and compares them to the designed values quantitatively (Fig. 6.5B). This was then used to create a feedback loop that could assist in identifying design constraints and optimizing the printing process. After adjustments were made the printing process was repeated with significantly better printing outcomes. They found the mismatch of morphological parameters decreased from 40% to 7% (Wang, Xu, Luo, Zhou, & Si, 2018).

6.6 CURRENT LIMITATIONS

One of the main limitations with bioink printability relates to material homogeneity. We have identified clumping, overdeposition, underdeposition, print inaccuracies, clogging, and filament discontinuities as possible outcomes when nonhomogeneous material is printed (Gillispie, Prim, et al., 2020). It is especially challenging to achieve true material homogeneity when working with naturally derived materials which have a natural variance in their properties depending on isolation and processing methods. The impact of surface tension on printing outcomes is also poorly understood and warrants further research (Chakrabarti & Chaudhury, 2013; Gillispie, Prim, et al., 2020). Another limitation is the inherent tradeoffs between high viscosity and low viscosity bioinks. Typically, bioinks that are highly viscous exhibit poor extrudability but can maintain excellent shape fidelity as well as print accuracy. On the contrary, bioinks with low viscosity are easily extruded at the cost of poor shape fidelity and difficulty maintaining high print accuracy (Gillispie, Prim, et al., 2020). Printability outcomes such as printing accuracy, shape fidelity, and extrudability are typically discussed separately but they are not truly independent.

Current printability assessments are limited by the difficulty of determining whether material properties or printing parameters are mainly affecting the poor printability outcome. In many instances the material properties clearly result in filament spreading, collapse, or poor shape fidelity, while in other studies it is unclear what parameter was the ultimate cause of failure (Gillispie, Prim, et al., 2020). The common theme in bioprinting alters either printing pressure or feedrate to control the material deposition while never actually measuring or controlling the material quantity within a print (Gillispie, Prim, et al., 2020). This can often lead to over- or underdeposition which results in poor printability outcomes. Overall, there exists a need for a standardized method to evaluate printing outcomes due to the large number of variables that can be controlled within the printing session. If each research group uses a different method or printing conditions to analyze printability it will remain difficult to compare outcomes between studies.

6.7 Conclusion

The ability of hydrogels to achieve desired printing outcomes continues to be of high interest to the field. Researchers have many tools at their disposal to improve the printability of their bioinks, including chemical modifications, optimizing bioink composition, rheological properties, and printing parameters. One of the most challenging aspects of bioink design is the interrelatedness of each of these factors. Composition and chemistry influence the hydrogel's response to external forces

(rheological properties) and the window of operable printing conditions which may be appropriate to use. Even the optimal hydrogel will result in poor printing outcomes if the correct printing conditions are not utilized. Meanwhile, although many studies have looked at how chemical modifications influence the rheological properties of different hydrogels, the translation to printability is not always a direct one. It has been established that shear-thinning, yielding, and quick recovery/self-healing behaviors are desirable from a printability perspective. However, some bioinks may exhibit these behaviors yet still result in poor printability outcomes. Further research is needed to develop models that can predict printability as well as improve the predictability of these relationships and allow for more intentional design during the bioink development process.

By focusing on the various suggestions presented above, we are sure that continuing efforts will lead to great promise for the next stage of development of bioinks, allowing better and more accurate control of optimized rheological and mechanical properties. Finally, the advanced design criteria established by various chemical strategies will undoubtedly contribute to the future development of bioinks with practical applicability in clinical use.

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QUESTIONS

1. What are the basic requirements of a bioink in regards to supporting the 3D printing process?
2. How is shape fidelity of a bioink most frequently measured and what are the drawbacks of this?
3. What is rheology and why is it important for characterizing bioinks?
4. During bioprinting, what is the main mechanism of damage to cells? And, how to minimize the cell damage during the printing process?
5. Why are shear-thinning bioinks desirable for 3D bioprinting applications?
6. What are some possible outcomes of printing nonhomogenous materials?

References

- Abbadessa, A., Mouser, V. H. M., Blokzijl, M. M., Gawlitta, D., Dhert, W. J. A., Hennink, W. E., ... Vermonden, T. (2016). A synthetic thermosensitive hydrogel for cartilage bioprinting and its biofunctionalization with polysaccharides. *Biomacromolecules*, 17(6), 2137–2147.
- Aguado, B. A., Mulyasmita, W., Su, J., Lampe, K. J., & Heilshorn, S. C. (2012). Improving viability of stem cells during syringe needle flow through the design of hydrogel cell carriers. *Tissue Engineering. Part A*, 18(7–8), 806–815.
- Ahlfeld, T., Cidonio, G., Kilian, D., Duin, S., Akkineni, A. R., Dawson, J. I., ... Gelinsky, M. (2017). Development of a clay based bioink for 3D cell printing for skeletal application. *Biofabrication*, 9(3), 034103.

- Ahn, G., Min, K. H., Kim, C., Lee, J. S., Kang, D., Won, J. Y., ... Shim, J. H. (2017). Precise stacking of decellularized extracellular matrix based 3D cell-laden constructs by a 3D cell printing system equipped with heating modules. *Scientific Reports*, 7(1), 8624.
- Ali, M., Pr, A. K., Yoo, J. J., Zahran, F., Atala, A., & Lee, S. J. (2019). A photo-crosslinkable kidney ECM-derived bioink accelerates renal tissue formation. *Advanced Healthcare Materials*, 8(7), e1800992.
- Arenas-Herrera, J. E., Ko, I. K., Atala, A., & Yoo, J. J. (2013). Decellularization for whole organ bioengineering. *Biomedical Materials (Bristol, England)*, 8(1), 014106.
- Atala, A., Kasper, F. K., & Mikos, A. G. (2012). Engineering complex tissues. *Science Translational Medicine*, 4(160), 160rv12.
- Athirasala, A., Tahayeri, A., Thrivikraman, G., Franca, C. M., Monteiro, N., Tran, V., ... Bertassoni, L. E. (2018). A dentin-derived hydrogel bioink for 3D bioprinting of cell laden scaffolds for regenerative dentistry. *Biofabrication*, 10(2), 024101.
- Basu, S., Pacelli, S., Feng, Y., Lu, Q., Wang, J., & Paul, A. (2018). Harnessing the noncovalent interactions of DNA backbone with 2D silicate nanodisks to fabricate injectable therapeutic hydrogels. *ACS Nano*, 12(10), 9866–9880.
- Bendtsen, S. T., Quinnell, S. P., & Wei, M. (2017). Development of a novel alginate-polyvinyl alcohol-hydroxyapatite hydrogel for 3D bioprinting bone tissue engineered scaffolds. *Journal of Biomedical Materials Research. Part A*, 105(5), 1457–1468.
- Berebichez-Fridman, R., & Montero-Olvera, P. R. (2018). Sources and clinical applications of mesenchymal stem cells: State-of-the-art review. *Sultan Qaboos University Medical Journal [SQUMJ]*, 18(3).
- Billiet, T., Gevaert, E., De Schryver, T., Cornelissen, M., & Dubruel, P. (2014). The 3D printing of gelatin methacrylamide cell-laden tissue-engineered constructs with high cell viability. *Biomaterials*, 35(1), 49–62.
- Blaeser, A., Duarte Campos, D. F., Weber, M., Neuss, S., Theek, B., Fischer, H., & Jähnen-Dechent, W. (2013). Biofabrication under fluorocarbon: A novel freeform fabrication technique to generate high aspect ratio tissue-engineered constructs. *Bioresearch Open Access*, 2(5), 374–384.
- Chakrabarti, A., & Chaudhury, M. K. (2013). Direct measurement of the surface tension of a soft elastic hydrogel: Exploration of elastocapillary instability in adhesion. *Langmuir: The ACS Journal of Surfaces and Colloids*, 29(23), 6926–6935.
- Chang, C. C., Boland, E. D., Williams, S. K., & Hoying, J. B. (2011). Direct-write bioprinting three-dimensional biohybrid systems for future regenerative therapies. *Journal of Biomedical Materials Research. Part B, Applied Biomaterials*, 98(1), 160–170.
- Cheng, J., Lin, F., Liu, H., Yan, Y., Wang, X., Zhang, R., & Xiong, Z. (2008). Rheological properties of cell-hydrogel composites extruding through small-diameter tips. *Journal of Manufacturing Science and Engineering*, 130(2).
- Chimene, D., Lennox, K. K., Kaunas, R. R., & Gaharwar, A. K. (2016). Advanced bioinks for 3D printing: A materials science perspective. *Annals of Biomedical Engineering*, 44(6), 2090–2102.
- Chung, J. H. Y., Naficy, S., Yue, Z., Kapsa, R., Quigley, A., Moulton, S. E., & Wallace, G. G. (2013). Bio-ink properties and printability for extrusion printing living cells. *Biomaterials Science*, 1(7), 763–773.
- Cohen, D. L., Lo, W., Tsavaris, A., Peng, D., Lipson, H., & Bonassar, L. J. (2011). Increased mixing improves hydrogel homogeneity and quality of three-dimensional printed constructs. *Tissue Engineering. Part C, Methods*, 17(2), 239–248.
- Coussot, P. (2014). Yield stress fluid flows: A review of experimental data. *The Journal of Non-Newtonian Fluid Mechanics*, 211, 31–49.
- Dahal, E., Badal, A., Zidan, A., Alayoubi, A., Hagio, T., Glick, S., ... Ghammraoui, B. (2018). Stable gelatin-based phantom materials with tunable x-ray attenuation properties and 3D printability for x-ray imaging. *Physics in Medicine and Biology*, 63(9), 09NT01.

- Daly, A. C., Critchley, S. E., Rencsok, E. M., & Kelly, D. J. (2016). A comparison of different bioinks for 3D bioprinting of fibrocartilage and hyaline cartilage. *Biofabrication*, 8(4), 045002.
- Dávila, J. L., & d'Ávila, M. A. (2018). Rheological evaluation of laponite/alginate inks for 3D extrusion-based printing. *The International Journal of Advanced Manufacturing Technology*, 101(1–4), 675–686.
- Diamantides, N., Dugopolski, C., Blahut, E., Kennedy, S., & Bonassar, L. J. (2019). High density cell seeding affects the rheology and printability of collagen bioinks. *Biofabrication*, 11(4), 045016.
- Diamantides, N., Wang, L., Pruiksma, T., Siemiatkoski, J., Dugopolski, C., Shortkroff, S., . . . Bonassar, L. J. (2017). Correlating rheological properties and printability of collagen bioinks: The effects of riboflavin photocrosslinking and pH. *Biofabrication*, 9(3), 034102.
- Duan, B., Hockaday, L. A., Kang, K. H., & Butcher, J. T. (2013). 3D bioprinting of heterogeneous aortic valve conduits with alginate/gelatin hydrogels. *Journal of Biomedical Materials Research. Part A*, 101(5), 1255–1264.
- Duan, B., Kapetanovic, E., Hockaday, L. A., & Butcher, J. T. (2014). Three-dimensional printed trileaflet valve conduits using biological hydrogels and human valve interstitial cells. *Acta Biomaterialia*, 10(5), 1836–1846.
- Duarte Campos, D. F., Blaeser, A., Korsten, A., Neuss, S., Jakel, J., Vogt, M., & Fischer, H. (2015). The stiffness and structure of three-dimensional printed hydrogels direct the differentiation of mesenchymal stromal cells toward adipogenic and osteogenic lineages. *Tissue Engineering. Part A*, 21(3–4), 740–756.
- Duty, C., Ajinjeru, C., Kishore, V., Compton, B., Hmeidat, N., Chen, X., . . . Kunc, V. (2018). What makes a material printable? A viscoelastic model for extrusion-based 3D printing of polymers. *Journal of Manufacturing Processes*, 35, 526–537.
- Gao, Q., Zhao, H. M., Yang, F. F., Fu, J. Z., & He, Y. (2018). Practical laboratory methods for 3D bioprinting. *3D Bioprinting for Reconstructive Surgery*, 7–32.
- Gao, T., Gillispie, G. J., Copus, J. S., Pr, A. K., Seol, Y. J., Atala, A., . . . Lee, S. J. (2018). Optimization of gelatin–alginate composite bioink printability using rheological parameters: A systematic approach. *Biofabrication*, 10(3), 034106.
- Gillispie, G., Prim, P., Copus, J., Fisher, J., Mikos, A. G., Yoo, J. J., . . . Lee, S. J. (2020). Assessment methodologies for extrusion-based bioink printability. *Biofabrication*, 12(2), 022003.
- Gillispie, G. J., Han, A., Uzun-Per, M., Fisher, J., Mikos, A. G., Niazi, M. K. K., . . . Atala, A. (2020). The influence of printing parameters and cell density on bioink printing outcomes. *Tissue Engineering. Part A*, 26(23–24), 1349–1358.
- Gillispie, G. J., Park, J., Copus, J. S., Pallickaveedu Rajan Asari, A. K., Yoo, J. J., Atala, A., & Lee, S. J. (2019). Three-dimensional tissue and organ printing in regenerative medicine. *Principles of Regenerative Medicine*, 831–852.
- Giuseppe, M. D., Law, N., Webb, B., Macrae, R. A., Liew, L. J., Sercombe, T. B., . . . Doyle, B. J. (2018). Mechanical behaviour of alginate–gelatin hydrogels for 3D bioprinting. *Journal of the Mechanical Behavior of Biomedical Materials*, 79, 150–157.
- Gohl, J., Markstedt, K., Mark, A., Hakansson, K., Gatenholm, P., & Edelvik, F. (2018). Simulations of 3D bioprinting: predicting bioprintability of nanofibrillar inks. *Biofabrication*, 10(3), 034105.
- Gopinathan, J., & Noh, I. (2018). Recent trends in bioinks for 3D printing. *Biomaterials Research*, 22, 11.
- Groll, J., Burdick, J. A., Cho, D. W., Derby, B., Gelinsky, M., Heilshorn, S. C., . . . Woodfield, T. B. F. (2018). A definition of bioinks and their distinction from biomaterial inks. *Biofabrication*, 11(1), 013001.
- Guvendiren, M., Molde, J., Soares, R. M., & Kohn, J. (2016). Designing biomaterials for 3D printing. *ACS Biomaterials Science & Engineering*, 2(10), 1679–1693.
- Habib, A., Sathish, V., Mallik, S., & Khoda, B. (2018). 3D Printability of alginate-carboxymethyl cellulose hydrogel. *Materials (Basel)*, 11(3).
- Habib, M. A., & Khoda, B. (2018). Development of clay based novel bio-ink for 3D bio-printing process. *Procedia Manufacturing*, 26, 846–856.

- He, Y., Yang, F., Zhao, H., Gao, Q., Xia, B., & Fu, J. (2016). Research on the printability of hydrogels in 3D bioprinting. *Scientific Reports*, 6(1), 29977.
- Holzl, K., Lin, S., Tytgat, L., Van Vlierberghe, S., Gu, L., & Ovsianikov, A. (2016). Bioink properties before, during and after 3D bioprinting. *Biofabrication*, 8(3), 032002.
- Hospodiuk, M., Dey, M., Sosnoski, D., & Ozbolat, I. T. (2017). The bioink: A comprehensive review on bioprintable materials. *Biotechnology Advances*, 35(2), 217–239.
- Jessop, Z. M., Al-Sabah, A., Gao, N., Kyle, S., Thomas, B., Badiei, N., . . . Whitaker, I. S. (2019). Printability of pulp derived crystal, fibril and blend nanocellulose-alginate bioinks for extrusion 3D bioprinting. *Biofabrication*, 11(4), 045006.
- Jia, J., Richards, D. J., Pollard, S., Tan, Y., Rodriguez, J., Visconti, R. P., . . . Mei, Y. (2014). Engineering alginate as bioink for bioprinting. *Acta Biomaterialia*, 10(10), 4323–4331.
- Jiang, D., Xue, Q., Liu, Z., Han, J., & Wu, X. (2017). Novel anti-algal nanocomposite hydrogels based on thiol/acetyl thioester groups chelating with silver nanoparticles. *New Journal of Chemistry*, 41(1), 271–277.
- Jin, Y., Chai, W., & Huang, Y. (2017). Printability study of hydrogel solution extrusion in nanoclay yield-stress bath during printing-then-gelation biofabrication. *Materials Science and Engineering: C*, 80, 313–325.
- Jungst, T., Smolan, W., Schacht, K., Scheibel, T., & Groll, J. (2016). Strategies and molecular design criteria for 3D printable hydrogels. *Chemical Reviews*, 116(3), 1496–1539.
- Kang, H. W., Lee, S. J., Ko, I. K., Kengla, C., Yoo, J. J., & Atala, A. (2016). A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. *Nature Biotechnology*, 34(3), 312–319.
- Kang, K. H., Hockaday, L. A., & Butcher, J. T. (2013). Quantitative optimization of solid freeform deposition of aqueous hydrogels. *Biofabrication*, 5(3), 035001.
- Khalil, S., & Sun, W. (2007). Biopolymer deposition for freeform fabrication of hydrogel tissue constructs. *Materials Science and Engineering: C*, 27(3), 469–478.
- Kim, J. H., Yoo, J. J., & Lee, S. J. (2016). Three-dimensional cell-based bioprinting for soft tissue regeneration. *Journal of Tissue Engineering and Regenerative Medicine*, 13(6), 647–662.
- Kirchmayer, D. M., Gorkin, R., III, & in het Panhuis, M. (2015). An overview of the suitability of hydrogel-forming polymers for extrusion-based 3D-printing. *Journal of Materials Chemistry B*, 3(20), 4105–4117.
- Kiyotake, E. A., Douglas, A. W., Thomas, E. E., Nimmo, S. L., & Detamore, M. S. (2019). Development and quantitative characterization of the precursor rheology of hyaluronic acid hydrogels for bioprinting. *Acta Biomaterialia*, 95, 176–187.
- Klar, V., Pere, J., Turpeinen, T., Karki, P., Orelma, H., & Kuosmanen, P. (2019). Shape fidelity and structure of 3D printed high consistency nanocellulose. *Scientific Reports*, 9(1), 3822.
- Kopf, M., Campos, D. F., Blaeser, A., Sen, K. S., & Fischer, H. (2016). A tailored three-dimensionally printable agarose-collagen blend allows encapsulation, spreading, and attachment of human umbilical artery smooth muscle cells. *Biofabrication*, 8(2), 025011.
- Kraut, G., Yenchesky, L., Prieto, F., Tovar, G. E. M., & Southan, A. (2017). Influence of shear thinning and material flow on robotic dispensing of poly(ethylene glycol) diacrylate/poloxamer 407 hydrogels. *Journal of Applied Polymer Science*, 134(29).
- Kyle, S., Jessop, Z. M., Al-Sabah, A., & Whitaker, I. S. (2017). ‘Printability’ of candidate biomaterials for extrusion based 3D printing: State-of-the-art. *Advanced Healthcare Materials*, 6(16).
- Kyle, S., Jessop, Z. M., Tarassoli, S. P., Al-Sabah, A., & Whitaker, I. S. (2018). Assessing printability of bioinks. *3D Bioprinting for Reconstructive Surgery*, 173–189.
- Laronda, M. M., Rutz, A. L., Xiao, S., Whelan, K. A., Duncan, F. E., Roth, E. W., . . . Shah, R. N. (2017). A bioprosthetic ovary created using 3D printed microporous scaffolds restores ovarian function in sterilized mice. *Nature Communications*, 8, 15261.

- Law, N., Doney, B., Glover, H., Qin, Y., Aman, Z. M., Sercombe, T. B., ... Doyle, B. J. (2018). Characterisation of hyaluronic acid methylcellulose hydrogels for 3D bioprinting. *Journal of the Mechanical Behavior of Biomedical Materials*, 77, 389–399.
- Lee, B. H., Lum, N., Seow, L. Y., Lim, P. Q., & Tan, L. P. (2016). Synthesis and characterization of types A and B gelatin methacryloyl for bioink applications. *Materials (Basel)*, 9(10).
- Lee, D. Y., Lee, H., Kim, Y., Yoo, S. Y., Chung, W. J., & Kim, G. (2016). Phage as versatile nanoink for printing 3-D cell-laden scaffolds. *Acta Biomaterialia*, 29, 112–124.
- Lee, J. M., Ng, W. L., & Yeong, W. Y. (2019). Resolution and shape in bioprinting: Strategizing towards complex tissue and organ printing. *Applied Physics Reviews*, 6(1).
- Lee, J. M., & Yeong, W. Y. (2015). A preliminary model of time-pressure dispensing system for bioprinting based on printing and material parameters. *Virtual and Physical Prototyping*, 10(1), 3–8.
- Lee, J. S., Kim, B. S., Seo, D., Park, J. H., & Cho, D. W. (2017). Three-dimensional cell printing of large-volume tissues: Application to ear regeneration. *Tissue Engineering. Part C, Methods*, 23(3), 136–145.
- Lee, S. C., Gillispie, G., Prim, P., & Lee, S. J. (2020). Physical and chemical factors influencing the printability of hydrogel-based extrusion bioinks. *Chemical Reviews*, 120(19), 10834–10886.
- Li, H., Liu, S., & Lin, L. (2016). Rheological study on 3D printability of alginate hydrogel and effect of graphene oxide. *International Journal of Bioprinting*, 2(2), 13.
- Li, M., Tian, X., Zhu, N., Schreyer, D. J., & Chen, X. (2010). Modeling process-induced cell damage in the bioprinting process. *Tissue Engineering. Part C, Methods*, 16(3), 533–542.
- Li, Z., Huang, S., Liu, Y. F., Yao, B., Hu, T., Shi, H. G., ... Fu, X. B. (2018). Tuning alginate–gelatin bioink properties by varying solvent and their impact on stem cell behavior. *Scientific Reports*, 8(1), 8020.
- Lopez, A., Marquette, C. A., & Courtial, E.-J. (2021). FingerMap: A new approach to predict soft material 3D objects printability. *Progress in Additive Manufacturing*, 6(1), 53–62.
- Maisonneuve, B. G. C., Roux, D. C. D., Thorn, P., & Cooper-White, J. J. (2013). Effects of cell density and biomacromolecule addition on the flow behavior of concentrated mesenchymal cell suspensions. *Biomacromolecules*, 14(12), 4388–4397.
- Malda, J., Visser, J., Melchels, F. P., Jüngst, T., Hennink, W. E., Dhert, W. J. A., ... Huttmacher, D. W. (2013). 25th anniversary article: Engineering hydrogels for biofabrication. *Advanced Materials*, 25(36), 5011–5028.
- Mandrycky, C., Wang, Z., Kim, K., & Kim, D.-H. (2016). 3D bioprinting for engineering complex tissues. *Biotechnology Advances*, 34(4), 422–434.
- Markstedt, K., Escalante, A., Toriz, G., & Gatenholm, P. (2017). Biomimetic inks based on cellulose nanofibrils and cross-linkable xylans for 3D printing. *ACS Applied Materials & Interfaces*, 9(46), 40878–40886.
- Markstedt, K., Mantas, A., Tournier, I., Martinez Avila, H., Hagg, D., & Gatenholm, P. (2015). 3D bioprinting human chondrocytes with nanocellulose-alginate bioink for cartilage tissue engineering applications. *Biomacromolecules*, 16(5), 1489–1496.
- Melchels, F. P. W., Dhert, W. J. A., Huttmacher, D. W., & Malda, J. (2014). Development and characterisation of a new bioink for additive tissue manufacturing. *Journal of Materials Chemistry B*, 2(16), 2282–2289.
- Moller, P. C. F., Fall, A., & Bonn, D. (2009). Origin of apparent viscosity in yield stress fluids below yielding. *Europhysics Letters*, 87(3).
- Moroni, L., Burdick, J. A., Highley, C., Lee, S. J., Morimoto, Y., Takeuchi, S., & Yoo, J. J. (2018). Biofabrication strategies for 3D *in vitro* models and regenerative medicine. *Nature Review Materials*, 3(5), 21–37.
- Mouser, V. H., Melchels, F. P., Visser, J., Dhert, W. J., Gawlitta, D., & Malda, J. (2016). Yield stress determines bioprintability of hydrogels based on gelatin-methacryloyl and gellan gum for cartilage bioprinting. *Biofabrication*, 8(3), 035003.
- Muller, M., Becher, J., Schnabelrauch, M., & Zenobi-Wong, M. (2015). Nanostructured pluronic hydrogels as bioinks for 3D bioprinting. *Biofabrication*, 7(3), 035006.

- Muller, M., Ozturk, E., Arlov, O., Gatenholm, P., & Zenobi-Wong, M. (2017). Alginate sulfate-nanocellulose bioinks for cartilage bioprinting applications. *Annals of Biomedical Engineering*, 45(1), 210–223.
- Murphy, S. V., Skardal, A., & Atala, A. (2013). Evaluation of hydrogels for bio-printing applications. *Journal of Biomedical Materials Research. Part A*, 101(1), 272–284.
- Nadgorny, M., Xiao, Z., & Connal, L. A. (2017). 2D and 3D-printing of self-healing gels: design and extrusion of self-rolling objects. *Molecular Systems Design & Engineering*, 2(3), 283–292.
- Ng, W. L., Yeong, W. Y., & Naing, M. W. (2016). Polyelectrolyte gelatin-chitosan hydrogel optimized for 3D bioprinting in skin tissue engineering. *International Journal of Bioprinting*, 2(1), 53–62.
- Ouyang, L., Yao, R., Zhao, Y., & Sun, W. (2016). Effect of bioink properties on printability and cell viability for 3D bioplotting of embryonic stem cells. *Biofabrication*, 8(3), 035020.
- Oyen, M. L. (2014). Mechanical characterisation of hydrogel materials. *International Materials Reviews*, 59(1), 44–59.
- Ozbolat, I. T., & Hospodiuk, M. (2016). Current advances and future perspectives in extrusion-based bioprinting. *Biomaterials*, 76, 321–343.
- Panwar, A., & Tan, L. (2016). Current status of bioinks for micro-extrusion-based 3D bioprinting. *Molecules (Basel, Switzerland)*, 21(6).
- Paxton, N., Smolan, W., Bock, T., Melchels, F., Groll, J., & Jungst, T. (2017). Proposal to assess printability of bioinks for extrusion-based bioprinting and evaluation of rheological properties governing bioprintability. *Biofabrication*, 9(4), 044107.
- Peak, C. W., Stein, J., Gold, K. A., & Gaharwar, A. K. (2018). Nanoengineered colloidal inks for 3D bioprinting. *Langmuir: The ACS Journal of Surfaces and Colloids*, 34(3), 917–925.
- Petta, D., Grijpma, D. W., Alini, M., Eglin, D., & D'Este, M. (2018). Three-dimensional printing of a tyramine hyaluronan derivative with double gelation mechanism for independent tuning of shear thinning and post-printing curing. *ACS Biomaterials Science & Engineering*, 4, 3088–3098. Available from <https://doi.org/10.1021/acsbomaterials.8b00416>.
- Picout, D. R., & Ross-Murphy, S. B. (2003). Rheology of biopolymer solutions and gels. *The Scientific World Journal*, 3, 105–121.
- Ribeiro, A., Blokzijl, M. M., Levato, R., Visser, C. W., Castilho, M., Hennink, W. E., ... Malda, J. (2017). Assessing bioink shape fidelity to aid material development in 3D bioprinting. *Biofabrication*, 10(1), 014102.
- Roehm, K. D., & Madhally, S. V. (2017). Bioprinted chitosan-gelatin thermosensitive hydrogels using an inexpensive 3D printer. *Biofabrication*, 10(1), 015002.
- Rutz, A. L., Hyland, K. E., Jakus, A. E., Burghardt, W. R., & Shah, R. N. (2015). A multimaterial bioink method for 3D printing tunable, cell-compatible hydrogels. *Advanced Materials*, 27(9), 1607–1614.
- Sachs, P. C., Mollica, P. A., & Bruno, R. D. (2017). Tissue specific microenvironments: A key tool for tissue engineering and regenerative medicine. *Journal of Biological Engineering*, 11(1).
- Sarker, M., & Chen, X. B. (2017). Modeling the flow behavior and flow rate of medium viscosity alginate for scaffold fabrication with a three-dimensional bioplotter. *The Journal of Manufacturing Science and Engineering*, 139(8).
- Schacht, K., Jungst, T., Schweinlin, M., Ewald, A., Groll, J., & Scheibel, T. (2015). Biofabrication of cell-loaded 3D spider silk constructs. *Angewandte Chemie International Edition*, 54(9), 2816–2820. (in English).
- Schuurman, W., Levett, P. A., Pot, M. W., van Weeren, P. R., Dhert, W. J., Hutmacher, D. W., ... Malda, J. (2013). Gelatin-methacrylamide hydrogels as potential biomaterials for fabrication of tissue-engineered cartilage constructs. *Macromolecular Bioscience*, 13(5), 551–561.
- Schwab, A., Levato, R., D'Este, M., Piluso, S., Eglin, D., & Malda, J. (2020). Printability and shape fidelity of bioinks in 3D bioprinting. *Chemical Reviews*, 120(19), 11028–11055.

- Shin, S., Park, S., Park, M., Jeong, E., Na, K., Youn, H. J., & Hyun, J. (2017). Cellulose nanofibers for the enhancement of printability of low viscosity gelatin derivatives. *BioResources*, 12(2).
- Smith, P. T., Basu, A., Saha, A., & Nelson, A. (2018). Chemical modification and printability of shear-thinning hydrogel inks for direct-write 3D printing. *Polymer*, 152, 42–50.
- Suntornnond, R., Tan, E., An, J., & Chua, C. (2016). A mathematical model on the resolution of extrusion bioprinting for the development of new bioinks. *Materials*, 9(9).
- Suntornnond, R., Tan, E. Y. S., An, J., & Chua, C. K. (2017). A highly printable and biocompatible hydrogel composite for direct printing of soft and perfusable vasculature-like structures. *Scientific Reports*, 7(1), 16902.
- Sweeney, M., Campbell, L. L., Hanson, J., Pantoya, M. L., & Christopher, G. F. (2017). Characterizing the feasibility of processing wet granular materials to improve rheology for 3D printing. *The Journal of Materials Science*, 52(22), 13040–13053.
- Tabriz, A. G., Hermida, M. A., Leslie, N. R., & Shu, W. (2015). Three-dimensional bioprinting of complex cell laden alginate hydrogel structures. *Biofabrication*, 7(4), 045012.
- Therriault, D., White, S. R., & Lewis, J. A. (2007). Rheological behavior of fugitive organic inks for direct-write assembly. *Applied Rheology*, 17(1), 10112-1–10112-8.
- Tirella, A., Orsini, A., Vozzi, G., & Ahluwalia, A. (2009). A phase diagram for microfabrication of geometrically controlled hydrogel scaffolds. *Biofabrication*, 1(4), 045002.
- Vijayavenkataraman, S., Vialli, N., Fuh, J. Y. H., & Feng Lu, W. (2019). Conductive collagen/PPy-b-PCL hydrogel for bioprinting of neural tissue constructs. *International Journal of Bioprinting*, 5(2.1).
- Wang, L., Xu, M.-E., Luo, L., Zhou, Y., & Si, P. (2018). Iterative feedback bio-printing-derived cell-laden hydrogel scaffolds with optimal geometrical fidelity and cellular controllability. *Scientific Reports*, 8(1), 1–13.
- Wang, L., Zhang, L., Zhou, Q., & Luo, L. (2016). Automated quantitative Assessment of three-Ddimensional bioprinted hydrogel scaffolds using optical coherence tomography. *Biomedical Optics Express*, 7, 894–910. Available from <https://doi.org/10.1364/BOE.7.000894>.
- Wang, X., He, K., & Zhang, W. (2013). Optimizing the fabrication processes for manufacturing a hybrid hierarchical polyurethane–cell/hydrogel construct. *Journal of Bioactive and Compatible Polymers*, 28(4), 303–319.
- Webb, B., & Doyle, B. J. (2017). Parameter optimization for 3D bioprinting of hydrogels. *Bioprinting*, 8, 8–12.
- Wilson, S. A., Cross, L. M., Peak, C. W., & Gaharwar, A. K. (2017). Shear-thinning and thermo-reversible nanoengineered inks for 3D bioprinting. *ACS Applied Materials & Interfaces*, 9(50), 43449–43458.
- Wu, Y., Lin, Z. Y., Wenger, A. C., Tam, K. C., & Tang, X. (2018). 3D bioprinting of liver-mimetic construct with alginate/cellulose nanocrystal hybrid bioink. *Bioprinting*, 9, 1–6.
- Wust, S., Godla, M. E., Muller, R., & Hofmann, S. (2014). Tunable hydrogel composite with two-step processing in combination with innovative hardware upgrade for cell-based three-dimensional bioprinting. *Acta Biomaterialia*, 10(2), 630–640.
- Yan, K. C., Nair, K., & Sun, W. (2010). Three dimensional multi-scale modelling and analysis of cell damage in cell-encapsulated alginate constructs. *Journal of Biomechanics*, 43(6), 1031–1038.
- Zhao, Y., Li, Y., Mao, S., Sun, W., & Yao, R. (2015). The influence of printing parameters on cell survival rate and printability in microextrusion-based 3D cell printing technology. *Biofabrication*, 7(4), 045002.
- Zhu, F., Cheng, L., Yin, J., Wu, Z. L., Qian, J., Fu, J., & Zheng, Q. (2016). 3D printing of ultratough polyion complex hydrogels. *ACS Applied Materials & Interfaces*, 8(45), 31304–31310.

Hydrogels for Bioprinting

7

Jia Min Lee¹, Wai Cheung Ma^{1,2} and Wai Yee Yeong^{1,2}

¹*School of Mechanical and Aerospace Engineering, Nanyang Technological University, Singapore*

²*Singapore Centre for 3D Printing (SC3DP), School of Mechanical and Aerospace Engineering, Nanyang Technological University, Singapore*

7.1 HYDROGELS IN BIOPRINTING

Hydrogels have drawn significant attention in bioprinting and tissue engineering due to their high water contents. Network of hydrophilic polymer chains are crosslinked either through chemical or physical interaction. Hydrogel formed through chemical bonds, such as covalent bonds, are permanent and irreversible. Whereas, hydrogel formed through physical interactions, such as (1) hydrogen bonds, (2) ionic forces, (3) physical entanglements of individual polymer chains, (4) polymer crystallites, and (5) hydrophobic, are reversible under certain conditions. Alternatively, hydrogel can be formed through a combination of two or more of the above interactions. There are a number of ways to categorize hydrogels, based on preparation methods, ionic charge, or physicochemical structural features and are summarized in Table 7.1 (Ratner, Hoffman, Schoen, & Lemons, 2013). In this section, examples of hydrogels used are discussed based on its origin, that is, natural versus synthetic hydrogel. A third category that would be discussed is the use of designer polymers in hydrogel system. These engineered polymers are biologically inspired, where polymers are functionalized onto existing hydrogel systems or synthesizing the building blocks from a bottom-up approach.

7.1.1 NATURAL HYDROGEL

Natural hydrogel has been heavily utilized for tissue engineering because they usually already contain specific bioactive regions which make them have good cellular compatibility with the cells of interest. Also because they are usually produced via biological methods, they tend to have a more defined structure and with a monodispersed molecular weight. They are usually biodegradable and with mechanical properties similar to that of the cell's natural ECM. However, they have issues regarding immunogenicity and have relatively unstable as compared to their synthetic counterparts. They also vary in properties between species, tissue, and batch of production. Also due to their complex composition, characterizing their innate properties is relatively difficult.

Table 7.1 Classification of hydrogels (Ratner et al., 2013).

Method of preparation	Ionic hydrogel	Physiochemical structural features	Complexation hydrogel
i) Homopolymer—one type of hydrophilic monomer unit ii) Copolymer—crosslinking two comonomer units with at least on hydrophilic monomer iii) Multi polymer—three of more comonomers iv) Interpenetrating network—Intermeshed network of monomer with crosslinked hydrogel of different mechanism	i) Neutral—Uncharged ii) Anionic—Negative charges only iii) Cationic—Positive charges only iv) Ampholytic—Positive and negative charges	i) Amorphous—covalent crosslinks—random arrangement ii) Semicrystalline—May or may not have covalent crosslinks—self-assembled, ordered macromolecular chains	i) Hydrogen bonds ii) Hydrophobic group associations iii) Affinity complexes—heterodimers—biotin/streptavidin—antibody/antigen—conA/glucose—PDLA/PLLA stereocomplexes—cyclodextrin inclusion complexes

7.1.1.1 Collagen

Collagen, a well-known protein, has been extensively used in biomedical applications as it is the main component of natural ECM and also the most abundant protein in mammalian tissues (Lee, Singla, & Lee, 2001). Although a large number of natural and synthetic polymers are used as biomaterials, the characteristics of collagen are distinct from those of synthetic polymers largely in its mode of interaction in the body. More than 20 different types of collagen have been found but their basic structures are the same, namely, consisting of three polypeptide chains. These three chains wrap around one another, thereby creating a three-stranded rope structure. The strands are held together by covalent bonds and hydrogen. Stable collagen fibers can initiatively form as a result of strands self-aggregating. Furthermore, the mechanical properties of collagen fibers can be enhanced by introducing chemical crosslinkers such as carbodiimide and glutaraldehyde (Lee, Grodzinsky, & Spector, 2001; Park, Park, Kim, Song, & Suh, 2002). Collagen is naturally degraded by metalloproteases and thus the degradation process can be locally controlled by cells in the engineered tissue.

7.1.1.2 Gelatin

Gelatin, a protein-based polymer, is derived through partial hydrolysis of collagen. Chemical pretreatment followed by heat treatment disorganizes collagen protein structure resulting in helix-to-coil transition and conversion into soluble gelatin. Gelatin has a lower antigenicity as compared to collagen (Ratner et al., 2013). Gelatin undergoes gelation during a change in temperature. However, gelatin has been modified to photopolymerizable hydrogel by addition of methylacrylate group (GelMA). Several studies have used GelMA for bioprinting (Billiet, Gevaert, De Schryver, Cornelissen, & Dubruel, 2014; Duan, Kapetanovic, Hockaday, & Butcher, 2013; Soman, Chung, Zhang, & Chen, 2013; Visser et al., 2013).

7.1.1.3 Fibrin

Fibrin is a biopolymer formed through thrombin-mediated cleavage of fibrinogen which resulted in self-associated, insoluble fibrin monomer (Ahmed, Dare, & Hincke, 2008). This naturally occurring material has been used as injectable scaffolds and cell delivery vehicles (Dare, Vascotto, Carlsson, Hincke, & Griffith, 2007). The major advantage is that fibrinogen can be autologously obtained from the plasma, which reduces the risks of a foreign body reaction. Moreover, fibrin has good adhesion capabilities. Generally, fibrin is used as glue to control bleeding and adhere tissues in surgery. In addition, it has also shown promises in skin grafts and the delivery of exogenous growth factors to reduce wound healing time.

7.1.1.4 Alginate

Alginate, a natural polymeric material, is derived from brown seaweed and bacteria. It has shown wide range of medical applications in drug stabilization and delivery, and cell encapsulation. It is a linear polysaccharide copolymer of α -L-guluronic acid (G) and (1–4)-linked β -D-mannuronic acid (M) monomers (see Figure 7.1). The G and M monomers are distributed sequentially in either alternating or repeating blocks (Donati & Paoletti, 2009; Johnson, Craig, & Mercer, 1997). Under neutral pH, alginate is a polyanion. It has low toxicity and gels under gentle conditions. The species, location, and age of seaweed are the influential factors that determine the amount and distribution of each monomer. Alginate gels are formed as a result of divalent cations such as Ba^{2+} , Ca^{2+} , or Sr^{2+} cooperatively interacting with G monomer blocks to generate ionic bridges between different polymer chains. By changing and adjusting the G and M ratio together with molecular weight of the polymer chain, the crosslinking density and the resulting mechanical properties can be easily manipulated. It is noted that ionically crosslinked alginate hydrogels undergo slow and uncontrolled dissolution rather than following a specific degradation trend. Mass is gradually lost through ion exchange of calcium and consequently individual chains are dissociated, leading to the loss of mechanical stiffness over time.

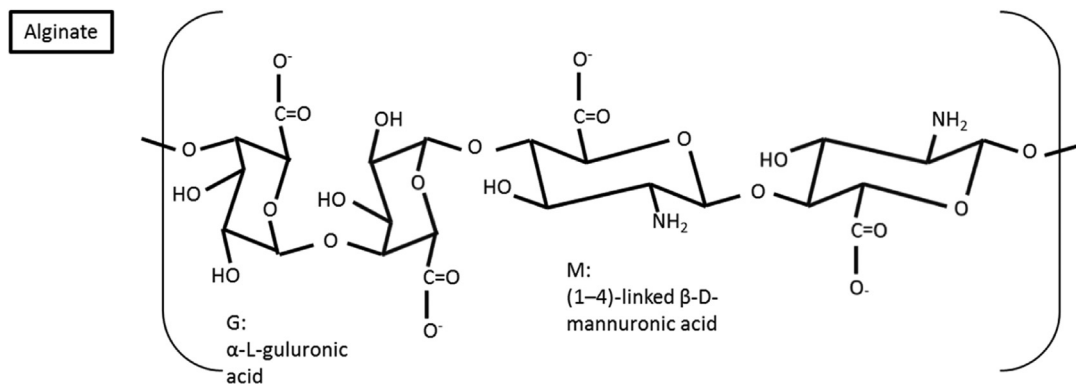


FIGURE 7.1

Chemical structure of alginate.

7.1.1.5 Chitosan and Chitin

Chitin and chitosan has shown to be useful in wound dressing and drug delivery. Chitin is a copolymer of *N*-acetyl-glucosamine and *N*-glucosamine units randomly or block distributed throughout the biopolymer chain depending on the processing method used to derive the biopolymer (Khor & Lim, 2003). The biopolymer is termed chitin if the number of *N*-acetyl-glucosamine units is higher than 50%. Otherwise, the biopolymer is termed chitosan. Chitin and chitosan are commercially obtained from shellfish sources such as shrimps and crabs.

Chitosan is a variant of chitin, a partially deacetylated counterpart. Figure 7.2 depicts the difference in molecular structure of chitin and chitosan. Chitosan is degradable by enzymes in humans and is structurally similar to naturally occurring glycosaminoglycans. Chitosan is soluble in dilute acids and once dissolved, it can be gelled by extruding the solution into a nonsolvent or increasing the pH value. Chitosan is degraded by lysozyme and the degradation kinetics is inversely proportional to the degree of crystallinity. Its degraded product includes chitosan with lower molecular weight, *N*-acetyl-D-glucosamine residues, and chitoglignomers (Ren, Yi, Wang, & Ma, 2005).

7.1.1.6 Hyaluronic Acid

Hyaluronic acid (HA) is a linear polysaccharide consisting of a repeating disaccharide of (1–3) and (1–4)-linked β -D-glucuronic acid and *N*-acetyl- β -D-glucosamine units (Alberts et al., 1994), and the molecular structure is shown in Figure 7.3. It is a highly hydrated polyanionic macromolecule (Khan & Ahmad, 2013) and can be found in almost every mammalian tissue and fluid. It has been widely used in wound healing and the synovial fluid of joints. A number of methods can be used to form HA hydrogels, such as covalent crosslinking with hydrazide derivatives,

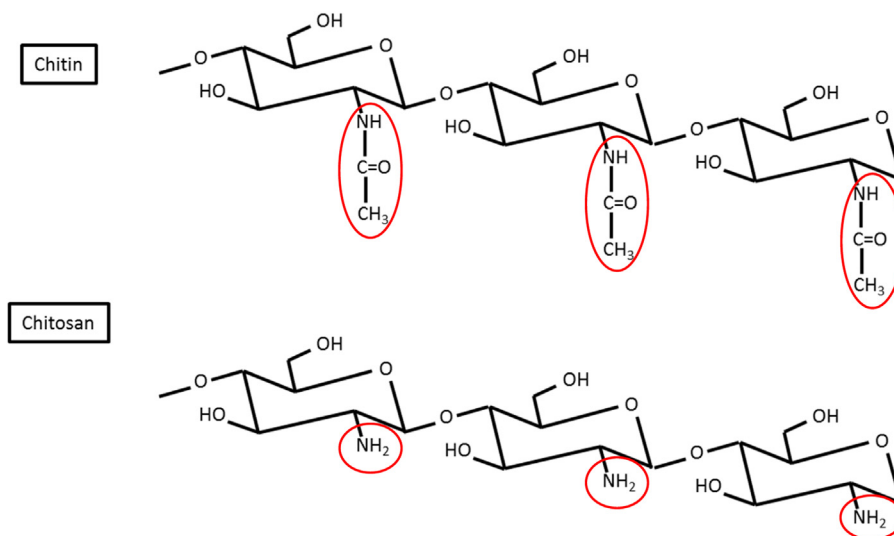
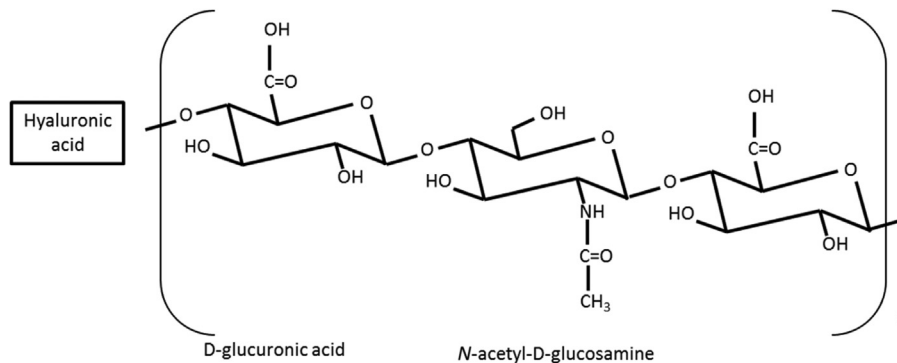


FIGURE 7.2

Difference between the chemical structure of chitin and chitosan.

**FIGURE 7.3**

Chemical structure of hyaluronic acid.

polyfunctional epoxides, carbodiimides, self-crosslink, and esterification (Collins & Birkinshaw, 2013). The reactive groups of HA ($-\text{OH}$ and $-\text{NHCOCH}_3$) provides crosslinking sites for hydrogel formation or can be modified for enhanced properties (Khan & Ahmad, 2013). HA is naturally degraded by hyaluronidase, which allows cells to regulate the clearance of the material locally.

7.1.1.7 Decellularized Extracellular Matrix

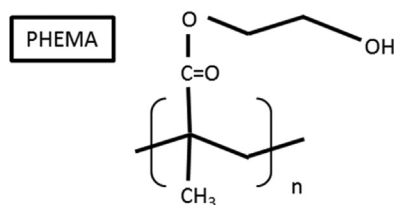
Decellularized extracellular matrices (dECMs) are biomaterials obtained from microtissues or organs after removal of the cellular components. This biological matrix has been described as an ideal scaffold material with its complex composition, vascular networks, and unique tissue-specific architecture (Taylor, Sampaio, Ferdous, Gobin, & Taite, 2018). dECM has been used in clinical settings and tissue engineering (Chun et al., 2010; Seo, Jung, & Kim, 2018). In bioprinting, dECMs are premixed with cells to form the bioink for printing (Choi et al., 2019; Ma et al., 2018).

7.1.2 SYNTHETIC HYDROGEL

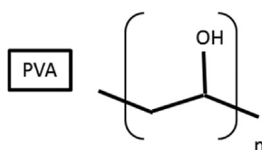
Synthetic hydrogels are some of the earliest biomaterials used as scaffolds in tissue engineering. The natural hydrogels presented in Section 7.1.1 are increasingly popular owing to their inherent biocompatibility. Comparatively, synthetic hydrogels have shown their benefits in the regenerative medicine field, including highly tunable and consistent properties, and easier large-scale production. This subsection presents some of the more commonly used synthetic hydrogels.

7.1.2.1 Poly(2-Hydroxyethyl Methacrylate)

Poly(2-hydroxyethyl methacrylate) (PHEMA) hydrogels have been used as implant materials since the late 1960s. It is synthesized using free radical precipitation polymerization of 2-hydroxyethyl methacrylate (Slaughter, Khurshid, Fisher, Khademhosseini, & Peppas, 2009). The resultant hydrogel is biologically inert but relatively weak. It is also a highly resistant material to protein adsorption and cell adhesion. However, it is found that PHEMA implants undergo delayed, episodic calcification *in vivo* (Belkas, Munro, Shoichet, Johnston, & Midha, 2005). PHEMA is a neutrally

**FIGURE 7.4**

Neutrally charged PHEMA monomers.

**FIGURE 7.5**

Neutrally charged PVA monomers.

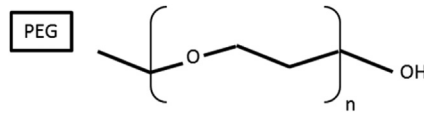
charged gel and the molecular structure of neutrally charged PHEMA repeat units is shown in [Figure 7.4](#).

7.1.2.2 Poly(vinyl alcohol)

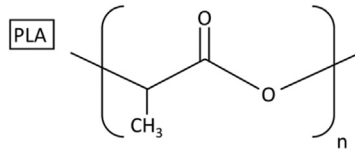
Poly(vinyl alcohol) (PVA; [Figure 7.5](#)) is prepared from the partial hydrolysis of poly(vinyl acetate). It can be crosslinked into a gel through either chemical or physical methods (*e.g.*, via treatment with monoaldehydes). PVA can also be photocured to fabricate hydrogels as an alternative to chemical crosslinking. PVA is similar to PHEMA in the aspect of having available pendant alcohol groups that function as attachment sites for biological molecules. PVA is an elastic material, which means it can induce matrix synthesis or cell orientation by enhancing the transmission of mechanical stimuli to seeded cells ([Schmedlen, Masters, & West, 2002](#)). PVA hydrogels are neutral and nonadhesive to proteins and cells. PVA hydrogels have a low friction coefficient and the structural properties are similar to natural cartilage. More importantly, they are generally stronger than most other synthetic gels, which makes them successful for avascular tissue. Furthermore, PVA can be copolymerized with poly(ethylene glycol) (PEG) to produce a biodegradable hydrogel which has a degradation rate faster than that of PEG hydrogels but slower than that of PVA homopolymer hydrogels.

7.1.2.3 Poly(ethylene glycol)

PEG ([Figure 7.6](#)) is a biocompatible and hydrophilic material, with properties that limit antigenicity, immunogenicity, cell adhesion, and protein binding ([Alcantar, Aydil, & Israelachvili, 2000](#)). PEG homopolymer is a polyether which can be polymerized from ethylene oxide by condensation. PEG is nonadsorptive due to the lack of protein binding sites on the polymer chain. As compared

**FIGURE 7.6**

Neutrally charged PEG monomers.

**FIGURE 7.7**

Neutrally charged poly(lactic acid) monomers.

to PHEMA and PVA, PEG does not have hydrogen bond donating groups, a feature that is critical in reducing protein binding.

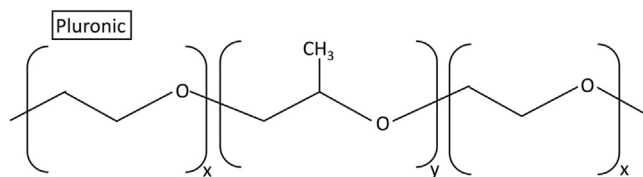
PEG hydrogels have been considered as one of the most successful synthetic hydrogels for tissue engineering applications. The ends of PEG polymer can be modified with either acrylates or methacrylate to form photocrosslinkable polyethylene glycol dimethacrylate (PEGDA) (Drury & Mooney, 2003). PEGDA is extensively used to encapsulate cells into scaffolds. Furthermore, PEG can act as a mediator for immobilizing the RGD sequence.

7.1.2.4 Poly(lactic acid)

Poly(lactic acid) (PLA) (Figure 7.7) is a polyester-based biocompatible and biodegradable material. The hydrophobic nature of the material reduces its interaction with cells when used for tissue engineering (Bee et al., 2018). Thus surface and bulk modification has been applied to increase cell adhesion for biomedical applications. PLA polymer can be synthesized through either direct polycondensation or ring opening polymerization of lactic acid and lactide monomers. PLA can be copolymerized with glycolic acid and PEG to modify its degradation rate and thermoresponsive properties (Basu, Kunduru, Doppalapudi, Domb, & Khan, 2016). PLA and its copolymers have been used for drug and protein release while serving as tissue scaffolds. The stimuli responsive properties of PLA copolymers further allow for targeted and controlled drug release to specific areas of the body when subjected to changes in the environment such as pH and temperature (Munim & Raza, 2019).

7.1.2.5 Poloxamers

Poloxamers are triblock copolymers which consist of two outer hydrophilic blocks with a central hydrophobic block. These polymers are more commonly known by their trade name of Pluronic (Figure 7.8). These hydrogels exhibit a temperature-based gelation process which works in reverse from most other hydrogels. For example Pluronic F127 forms a gel at room temperatures and liquefies when cooled to below 10°C. However, Pluronic has a low biocompatibility which is

**FIGURE 7.8**

General molecular structure of Pluronic.

unsuitable for long-term cell survival (Panwar & Tan, 2016). Due to these properties, Pluronic is more commonly used as a printed support material which can be easily removed. Pluronic can be used to help create channels or complex structures within another printed hydrogel (Kolesky et al., 2014). The construct can then be cooled and the Pluronic material can be easily washed off leaving the internal structures within the printed material.

7.1.3 BIOINSPIRED SYNTHETIC HYDROGEL

With advances in polymer synthesis, biomimicry functions have been added to hydrogel to illicit specific responses. There are several approaches in designing hydrogel systems with bioinspired features. The first approach adds biomimicry peptide sequences onto existing hydrogel systems to improve their mechanical and biological properties. This includes moieties that modulate adhesion (Choi, Kim, & Cha, 2021; Richardson et al., 2020), regulate immune response (He et al., 2019; Puthia et al., 2020), and promote angiogenesis (da Silva et al., 2018; Su, Satchell, Wertheim, & Shah, 2019). Some common peptides that have been conjugated onto the side chain or backbone of polymeric systems in view of eliciting biological response can be found in the following articles (Hosoyama, Lazurko, Muñoz, McTiernan, & Alarcon, 2019; Huettner, Dargaville, & Forget, 2018). Alternatively, biological sequences such as DNA have been conjugated to polymeric system such that the complementary DNA pair act as the crosslinking agent for hydrogel gelation (Dong et al., 2020; Du & Hill, 2019; Li et al., 2015; Zhao, Wang, Yan, & Liu, 2019).

The second approach is to synthesize monomers that are developed with the concept of hierarchical self-assembly. One such examples is collagen-mimicking peptides that contains Gly–X–Y trimeric sequences, which are characteristic of collagen (Chiang, Fu, & Horng, 2017; Pal, Jain, & Roy, 2020). Preliminary success in self-assembling short synthetic collagen peptides has been shown in these collagen-like sequences (Echalier et al., 2017; Lukomski & McNitt, 2020; Pal et al., 2020). Other than having ECM-like biomimetic architecture in the designer peptides, their degradation products are amino acids which eliminated the hazards of animal-related pathogens. However, due to the short sequence length in these biologically derived sequences, it is challenging to achieve gelation and higher order self-assembly as the alpha-helical structure was not stabilized. Another example is polymeric DNA hydrogel, where the oligonucleotides are the building blocks forming the hydrogel (Li, Tang, Geng, Luo, & Yang, 2019; Xing et al., 2011). These versatile molecular building blocks have the advantages of size-tunability, multivalency, and controllable symmetry which have potential for uses in nucleic acid–based diagnostics, drug delivery, and cell engineering (Dong et al., 2020).

7.2 CONSIDERATIONS FOR USING HYDROGEL IN BIOPRINTING

Bioprinting can be classified into three main processes, material extrusion, material jetting, and vat polymerization. Details regarding the available bioprinting technologies under the three processes can be found in the following article (Lee, Sing, Zhou, & Yeong, 2018). The following section will identify basic considerations of hydrogel used in bioprinting while further emphasizing material- and process-parameter considerations evaluated based on each bioprinting processes (Figure 7.9).

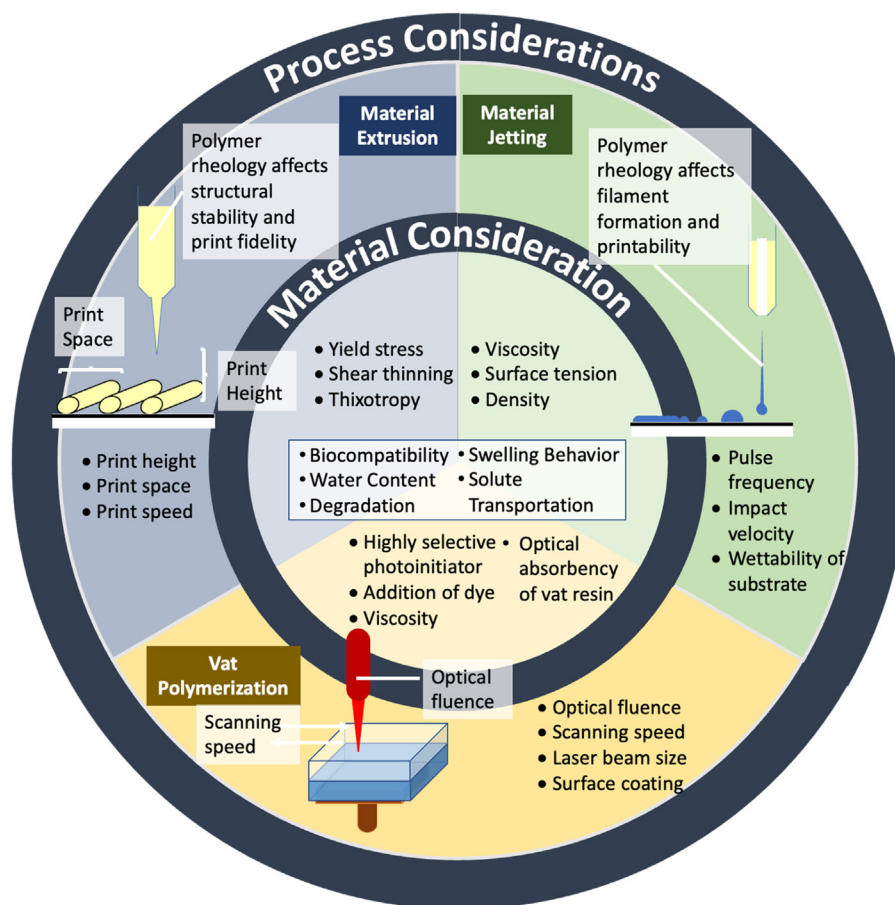


FIGURE 7.9

Material and process considerations in bioprinting based on different printing technologies.

7.2.1 GENERAL CONSIDERATION

7.2.1.1 Biocompatibility

Biocompatibility of the biomaterials is essentially the most important prerequisite for the material to be used as a scaffold. Biomaterials have to support cell attachment, migration, and other basic cellular functions (Chan & Leong, 2008). Chemicals involved during crosslinking of hydrogels also should not affect the cell viability (Nicodemus & Bryant, 2008). Some photoinitiators and monomers used during crosslinking of the hydrogels have been found to be toxic if they were left unreacted (Bryant, Nuttelman, & Anseth, 2000).

To evaluate biocompatibility, live/dead staining is usually employed after 24 hours to determine if the hydrogel and crosslinking process used would be cytocompatible to the cells. The result can be quantified using a microplate reader to determine the fluorescence intensity in which percentage of viable cells can be determined (Klouda, Hacker, Kretlow, & Mikos, 2009). MTS proliferative assay can be performed on alternate days to assess cytocompatibility and to determine the metabolic activity of the cells (Park et al., 2009).

7.2.1.2 Water Content

The amount of water in the hydrogel determines the adsorption rate and the diffusion of the solutes through the hydrogel. The water content in the hydrogel has to be controlled. If the water content in the hydrogel is too high, cell proliferation rate will deteriorate (Ogawa, Akazawa, & Tabata, 2010). Water can be bounded to the gel by two interactions. Firstly, the polar hydrophilic groups are hydrated and the network of polymer swells. Secondly, hydrophobic groups in the gels are exposed and water molecules are bound to the hydrophobic groups (Hoffman, 2002). Additional water is absorbed as free water which fills the space such as macropores and voids formed from between the chains. Water content in the hydrogel can be easily determined by either measuring the percentage change in the mass between the hydrogel and its dry form or by determining the light absorbance in the gel (Huglin & Yip, 1987; Nagaoka, Tanzawa, & Suzuki, 1990).

7.2.1.3 Swelling Behavior

Swelling and contraction of the bioprinted construct has to be considered and accounted for during the design of the tissue construct. The differences in the swelling properties between different hydrogels will cause complications such as restricted grafting of the layers and the overall uneven deformation of the construct (Murphy, Skardal, & Atala, 2013). Swelling behavior is also strongly dependent on the local environment such as pH, ionic strength, and solvent conditions. Swelling rates also affect the surface properties of the gel, the solute diffusion coefficient through these hydrogels, and the optical properties. The dynamic swelling and equilibrium in solutions of hydrogel governs the mechanical integrity of hydrogels. As described earlier, most water molecules within the hydrogel are bound by either by hydrophilic or hydrophobic groups. Most solutes can only diffuse into and through hydrogel from within the unbounded regions of the macropores and voids. Solutes which are chaotropic can diffuse through hydrogel by disturbing the interaction of the bounded water layers around the polymer chain.

The swelling behavior of the hydrogel can be thermodynamically described by the Flory-Huggins theory (Flory & Rehner, 1943). However, this theory does not account for the imperfections of the network or the finite volume of network chains and crosslinks in the gel. In this model,

the equilibrium of crosslinked polymer network swelled was described by the elastic forces of the polymer chains and the thermodynamic compatibility of the hydrogel polymer and the solvent, this is shown with the Gibbs free energy shown below. Swelling rates can be controlled by copolymerizing monomers of varying hydrophobicity.

$$\Delta G_{\text{total}} = \Delta G_{\text{elastic}} + \Delta G_{\text{mixing}}$$

However, for ionic gels, the total free energy contribution by the hydrogel would require the involvement of the ionic properties of the network.

$$\Delta G_{\text{total}} = \Delta G_{\text{elastic}} + \Delta G_{\text{mixing}} + \Delta G_{\text{ionic}}$$

At equilibrium potential, the net chemical potential between the solvent and the surrounding solution is zero. This zero net chemical potential balances both the elastic and mixing potential. Flory-Rhener theory evolves the expression that the hydrogel prepared in the absence of solvent should contain the following relationship:

$$\frac{1}{M_c} = \frac{1}{M_N} - \frac{\bar{v}(v/V_1)[\ln(1 - v_2) + v_{2,s} + \chi_1 v_{2,s}^2]}{v^{1/3} - \frac{v_2}{2}}$$

where $\bar{v}$ is the specific volume of the hydrophilic polymer, M_N is the primary molecular mass, M_c is the average molecular mass between the crosslinks, v_2 is the volume fraction of the polymer in the swollen mass, and V_1 is the molar volume of the solvent. The mixing term ΔG_{mixing} depends on the compatibility of the hydrophilic polymer and the solvent and is expressed as the polymer–solvent interaction parameter, χ_1 .

The equation is then modified by Peppas and Merrill for gels prepared in the presence of a solvent by including the changes in the elastic potential due to the solvent (Peppas & Merrill, 1977).

$$\frac{1}{M_c} = \frac{1}{M_N} - \frac{\bar{v}(v/V_1)[\ln(1 - v_{2,s}) + v_{2,s} + \chi_1 v_{2,s}^2]}{v_{2,r}[(v_{2,s}/v_{2,r})^{1/3} - (v_{2,s}/2v_{2,r})]}$$

Here, terms $v_{2,r}$ and $v_{2,s}$ show the volume fraction of the polymer in the relaxed and swollen state. The relaxed state refers to the crosslinked polymer without having any swelling occurring. Thus in the absence of a solvent, the volume fraction of the relaxed state is 1 simplifying the equation back to Flory-Rhener equation.

For ionic gels, two separate but equivalent expressions were derived for anionic and cationic gels (Brannon-Peppas & Peppas, 1991). These equations for average molecular weight between crosslinks require ionic strength, I and dissociation constants, K_a and K_b .

$$\left(\frac{V_1}{4I}\right)\left(\frac{v_{2,s}^2}{v}\right)\left(\frac{K_b}{10^{pH-14}-K_a}\right)^2 = [\ln(1 - v_{2,s}) + v_{2,s} + \chi_1 v_{2,s}^2] + \left(\frac{V_1}{vM_c}\right)\left(1 - \frac{2M_c}{M_N}\right)v_{2,r}\left[\left(\frac{v_{2,s}}{v_{2,r}}\right)^{1/3} - \frac{v_{2,s}}{2v_{2,r}}\right]$$

7.2.1.4 Solute Transportation

Another important aspect of designing hydrogel for tissue engineering is to develop an effective solute transport. This determines how cellular products, nutrients, and wastes are exchanged within the scaffold. The driving force of solute transportation is diffusion since the pores within the gels are too small for convection to play a significant role. In ionic gels, research has revealed that the hydrogel

mesh size and the culture conditions such as pH and temperature play an important role in diffusion (am Ende, Hariharan, & Peppas, 1995). In biological systems, the hydrophilic polymer and their solutes frequently interacts and ionizes with their surroundings, thus they become an important factor in determining the transport behavior. Further investigation has shown that this interaction tends to decrease transport rate of solutes into the hydrogel (Collins & Ramirez, 1979). Also, another research on the permeability of the hydrogel has also confirmed that the significance of this polymer–solute interaction and showed that it can be controlled by size exclusion (Gudeman & Peppas, 1995).

For nutrient transportation, an important measure for determining the maximum size of solute that can diffuse into the gel is its porosity. This can be predicted by the mesh size, ϵ , and the average linear distance between the crosslinks. To predict the mesh size, an equation containing the bond length of the polymer backbone, l (which is often 1.54 due to the carbon–carbon bone), the characteristic ratio, C_N , the average molecular weight between the crosslinks, M_c , the swollen polymer volume fraction, $v_{2,s}$, and the molecular weight of the monomer, M_r (Peppas, Hilt, Khademhosseini, & Langer, 2006; Tesoro, 1984).

$$\epsilon = (v_{2,s})^{-\frac{1}{3}} \left(\frac{2C_N M_c}{M_r} \right)^{1/2} l$$

In tissue engineered hydrogel, the mesh size of hydrogel has to include the effects of the solution containing salts, ions, and nutrients. Thus the swollen polymer fraction $v_{2,s}$ has to be revised to account for the solution used.

As all solute transport model involving hydrogel are based on diffusion, this enable the transport rate of solutes to be described using Fick's Law (Crank, 1979; Crank & Park, 1968). Fickian diffusion is, however, only applicable if the gel is amorphous. If the gel is significantly heterogenous, this law is no longer sufficient.

$$\frac{\partial c_i}{\partial t} = \nabla \cdot (D_{ig}(C_i) \nabla C_i)$$

where c_i is the concentration of the species i and D_{ig} is the concentration dependant coefficient of species i in the hydrogel.

Another model based on the free volume theory for water solute and polymer was developed by Peppas et al. to predict the dependency of the solute diffusion coefficient on solute size, mesh size, degree of swelling, and other structural properties of the hydrogel (Peppas & Reinhart, 1983).

$$\frac{D_{SM}}{D_{Sw}} = \frac{k_1(M_c - M_{C^*})}{M_N - M_{C^*}} \exp\left(-\frac{K_2 r_s^2}{Q_M - 1}\right)$$

where D_{SM} and D_{Sw} are the diffusion coefficients of the solutes in the membrane and solvent, respectively. This ratio is also known as the normalized diffusion coefficient. k_1 and k_2 are the structural parameters of the polymer–water complex. M_{C^*} is the average critical molecular weight between the crosslinks at which diffusion is excluded. r_s is the Stokes hydrodynamic radius of the solute and Q_M is the degree of swelling of the hydrogel. This experiment was validated using PVA membrane.

7.2.1.5 Degradation

The degradation rate of a material is usually matched with the rate of tissue regeneration (Dhandayuthapani, Yoshida, Maekawa, & Sakthi Kumar, 2011). This is to ensure that the scaffold

can still provide the necessary support for cells to regenerate completely. If the degradation rate is too short, the hydrogel support may be encapsulated by fibrous tissue when implanted into the body, blocking the overall cellular regeneration. If the degradation rate is too long, chronic inflammatory response may set in which in turn will cause greater damage to the body.

Unlike solid polymers, hydrogels undergo purely bulk degradation since they are hydrated within the structures. To control the degradation of these polymers, researchers have come up with hydrogels that have been copolymerized with peptides that are sensitive to enzymatic degradation (Drury & Mooney, 2003). One such research by Lei controlled the degradation with the use of PEG hydrogel that incorporates with matrix metalloproteinase sensitive peptides (Lei, Ng, & Segura, 2010). These peptides respond to local protease activity on the cell surface and degrade accordingly.

Determination of these changes in degradation can be characterized by determining changes in their weight, elastic modulus, and swelling degree with respect to their initial condition. The hydrolysis and enzymatic degradation of the hydrogel polymers reduces the efficiency of the crosslinking mechanism in the gel thus reducing the above properties of the gel (Lee, Bouhadir, & Mooney, 2004).

7.2.2 TECHNOLOGY SPECIFIC CONSIDERATION

The following section will discuss technology specific considerations looking at the variables in terms of material and process parameters for each of the three bioprinting processes, material extrusion, material jetting, and vat polymerization.

7.2.2.1 Material Extrusion

7.2.2.1.1 Material Consideration

Yield stress is the instantaneous pressure required to initiate the flow of material. For extrusion-based bioprinting, this property affects the minimum stress applied on the material before it is being dispensed. Yield stress will also help to keep the cells homogenous in the hydrogel reservoir (Malda et al., 2013). *Shear thinning* is ideal for an extrudable bioink. During dispensing, the shear force reorganizes the random polymer chains into an aligned conformation which reduces the hydrogel viscosity during the process (Yucel, Cebe, & Kaplan, 2009). The effect of shear thinning varies among the biomaterials with high molecular weight. *Thixotropy* behavior of the bioink determines whether the extruded material is able to retain its shape after material exits the dispensing nozzle. This time-dependent factor can be determined through a hysteresis loop test (Barbucci, Pasqui, Favaloro, & Panariello, 2008).

7.2.2.1.2 Process Consideration

Print outcomes are determined by factors such as *pressure input*, *print height*, *print space*, and *print speed* (Kang, Hockaday, & Butcher, 2013; Lee & Yeong, 2015; Tan, Yeong, 2015). The *printing pressure* affects the length, width, and height of printed layers. *Print height* is adjusted to prevent nozzle from disturbing previous print layers during traversing, while ensuring that the extruded bioink can reach the building platform on time. The print height is typically set to 75% of nozzle diameter.

7.2.2.2 Material Jetting

7.2.2.2.1 Material Consideration

Material properties that influence droplet formation process affect the print outcome in jetting-based bioprinting processes. Three key properties of bioinks that influence the ejection of droplets are the *viscosity*, *surface tension*, and *density* (Ng, Lee, Yeong, & Win Naing, 2017). In addition to the impact on droplet formation, surface tension and viscosity of material affects the spreading of droplet on substrate (Du, Xing, Cao, Yu, & Han, 2017).

7.2.2.2.2 Process Consideration

The interaction of droplet on substrate influences the droplet resolution. A substrate surface with higher contact angles would result in smaller droplet spreading due to slow energy dissipation (Mao, Kuhn, & Tran, 1997). A higher *impact velocity* leads to an increase in the maximum energy available for droplet rebound. Another process consideration essential for in droplet-based printing is the *coalescence of neighboring droplets*. Control over the spacing between droplets spacing determines whether individual drops, scallop lines, or linear lines are printed on the substrate (Du et al., 2017).

7.2.2.3 Vat Polymerization

7.2.2.3.1 Material Consideration

Viscosity of photoresin determines the refilling efficiency of the vat (Luo et al., 2016). *Highly selective photoinitiator* must be chosen to prevent unnecessary free radical crosslinking (Wang et al., 2018). *Light absorbency* of the vat resin influences the area at which photopolymerization occurs in the vat system. To improve on the z-resolution of printed construct in vat polymerization, photo-absorbers/dyes are added to limit the light penetration depth, preventing unwanted curing of the bioink (Han, Lu, Chester, & Lee, 2018; Khoon et al., 2018; Lee et al., 2015; Sakai et al., 2018).

7.2.2.3.2 Process Consideration

Process parameters such as *print stage velocity* and laser's optical power influences the print resolution in Wang et al. (2018). A higher stage velocity and lower optical power results in lesser exposure area that experience sufficient *optical fluence*, thus producing hydrogel with high resolution. It is necessary to surpass the threshold optical fluence to initiate sufficient photocrosslinking (Wang et al., 2018). Coating surface of the vat to minimize meniscus formation of the photopolymer ensures complete polymerization, thereby improving the shape and thickness of the construct (Curley, Jennings, & Moore, 2011).

7.3 STRATEGIES USED IN HYDROGEL-BASED BIOPRINTING

It is intrinsically difficult to bioprint soft materials into the air. Various printing and crosslinking strategies have been used to improve print fidelity and resolution (Lee & Yeong, 2016). The following section summarizes and categorizes the different approaches that have been used to improve print fidelity for hydrogel-based bioprinting.

7.3.1 TUNING RHEOLOGY OF BIOINK

It is essential to tune rheology of bioink according to the different processing technology used in bioprinting. A common approach for extrusion-based bioprinting processes is to tune bioink formulations to achieve desired viscosity, yield stress, and mechanical integrity for fast shear recovery during and after printing (Kesti et al., 2015; Malda et al., 2013; Panwar & Tan, 2016). Depending on the bioink's properties, adding enzyme crosslinker (Irvine et al., 2015), adjusting temperature of thermosensitive hydrogel (Lee & Yeong, 2014), and preexposure of photopolymer to UV light (Skardal, Zhang, McCoard, et al., 2010) form a semicrosslinked mixture with higher viscosity as compared to its precursor hydrogel. Adding thickening agents such as hydroxyapatite particles (Kesti et al., 2015; Li, Tian, & Chen, 2009), nanocellulose (Markstedt et al., 2015), xanthum gum (Tan, Yeong, 2015), and gellan gum (Kesti & Zenobi-Wong, 2015) increases the viscosity of the bioink.

Comparatively, jetting-based processes works with bioink of lower viscosity. For jetting of Newtonian fluid such as glycerol/water, properties such as viscosity, surface tension, and density of ink affect the printability (Jang, Kim, & Moon, 2009). Jetting of non-Newtonian fluid is controlled by capillary, viscous, elastic, and inertial effects (Xu, Zhang, Fu, & Huang, 2017). Xu et al. shown through variation in alginate concentration on the influence of relaxation time for the droplet formation in jetting process. Similarly, resins used in the vat polymerization processes have lower viscosity such that the hydrogel-precursors can flow during the refilling process after printing each successive layer. Additionally, it is critical for the vat resins to be nonthermosetting for high fidelity printing (Bertlein et al., 2017).

7.3.2 INDUCING CROSSLINKING DURING BIOPRINTING

Hydrogel with rapid gelation mechanism, such as sodium alginate with calcium ions and fibrinogen with thrombin, is commonly used as complementary pairs to induce crosslinking during bioprinting process. Coaxial printheads are designed such that crosslinking only occurs upon exiting the orifice (Costantini et al., 2017; Gao, He, Fu, Liu, & Ma, 2015; Nishiyama et al., 2008). Another approach is to sequentially deposit precursor hydrogel with its crosslinker (Faulkner-Jones et al., 2015; Lee et al., 2010; Li et al., 2015; Skardal et al., 2012; Tan & Yeong, 2014). With advancement in bioink development for bioprinting, a universal crosslinking strategy can be formulated such that multiple bioinks can be printed into a single, cohesive construct (Hull et al., 2020).

7.3.3 CROSSLINKING AFTER BIOPRINTING

In many cases, such printing approach uses mixture of hydrogels with multiple crosslinking mechanism. The approach uses a primary material that improves printability and shape fidelity during printing while the secondary material later undergoes crosslinking to provide structural fidelity after printing (Ahn, Lee, & Kim, 2013; Duan, Hockaday, Kang, & Butcher, 2013; Schuurman et al., 2013). For instance, GelMA/HA mixture showed enhanced printability as opposed to using GelMA alone (Schuurman et al., 2013). HA improves the viscosity of mixture while crosslinking of GelMA retains structure's integrity after printing.

7.3.4 BIOPRINTING WITH SUPPORT

Also termed as build/support configuration, the approach uses supporting materials to provide mechanical strength for hold the build materials (i.e., cells and/or hydrogel). The support material is either deposited sequentially with the build material (Kang et al., 2016; Kolesky et al., 2014; Miller et al., 2012; Skardal, Zhang, McCoard, et al., 2010; Wüst, Godla, Müller, & Hofmann, 2014) or exists as a bath (Choi et al., 2019; Hinton et al., 2015; Hull et al., 2020; Wu, DeConinck, & Lewis, 2011). Hydrogels with reversible crosslinking mechanism such as gelatin, Pluronic F127, and agarose are commonly used as the support material for easy retrieval of the build material.

7.3.5 HYBRID BIOPRINTING

The use of computer-assisted technology in bioprinting allows integration with several other fabrication processes. For instance, bioprinting techniques (such as extrusion and inkjet) have been integrated with melt-plotting and electrospinning apparatus to build multiscale parts (Kang et al., 2016; Lee, Ahn, Bonassar, Chun, & Kim, 2013; Pati et al., 2014; Schuurman et al., 2011; Shim, Kim, Park, Park, & Cho, 2011; Shim, Lee, Kim, & Cho, 2012; Xu, Zhao, et al., 2013). Thermoplastic polycaprolactone (PCL) and poly(lactic-co-glycolic acid) (PLGA) are commonly used as support material for hydrogel in hybrid printing. These thermoplastics are processed at high temperature which are not compatible to include cells in the plotting process. Hence, most hybrid bioprinting systems comprise of two components (1) dispensing thermoplastic—melt plotting system and (2) dispensing hydrogel/cell materials. As such, Table 7.2 summarizes some of the current biomaterials used in bioprinting along with the different printing techniques used. Instead, cells are deposited onto the thermoplastic using hydrogel as the carrier.

7.4 PERSPECTIVE AND OUTLOOK

Hydrogel-based bioprinting has been previously associated with engineered tissue lacking in structural integrity. Over the years, there have been advances in bioink development and developing printing strategies to improve on the shape fidelity of bioprinted construct. It is essential to understand and differentiate the term “printability” as a process-dependent factor, where a bioink that is printable using material jetting does not equvalate to it being printable using material extrusion. Thus printability assessment should be specific to each bioprinting processes. Material assessment criteria for each bioprinting process should be established and will provide a guidelines for researchers to evaluate and formulate suitable bioink. Strategies such as bioprinting with support have seen increased adoption by researchers to improve the integrity of print. However, there is still a need to improve on print resolution in bioprinting. With advances in integrating other technologies with bioprinting processes, finer resolution of bioprinted constructs can be tuned based on the fundamental units (i.e., single cell resolution and voxel-based material deposition). With deeper understanding over the physical processes involved in bioprinting, we would harness the ability to utilize bioprinting processes to our advantage. For instance, the shearing effect from extrusion-based bioprinting can be used to induce microstructural changes with enhanced performance (Lee & Yeong, 2020; Sydney Gladman, Matsumoto, Nuzzo, Mahadevan, & Lewis, 2016). It is

Table 7.2 Hydrogel used for bioprinting classified according to the type of material used.

Materials		Printing process	References
Natural hydrogel			
Alginate		Material extrusion	Ahn et al. (2013), Fedorovich and Wouter (2011), Huang, He, and Wang (2013), Lee et al. (2013), Ozbolat, Chen, and Yu (2014), Sawkins, Brown, Bonassar, Rose, and Shakesheff (2012), and Shim et al. (2012)
		Material jetting	Xu, Chai, Huang, and Markwald (2012) and Xu, Binder, et al. (2013)
Matrigel		Material extrusion	Snyder et al. (2011)
		Material jetting	Xu, Binder, et al. (2013)
Collagen		Material extrusion	Lee, Lee, and Polio (2009), Kim, Lee, and Kim (2016), and Rhee, Puetzer, Mason, Reinhart-King, and Bonassar (2016)
Gelatin		Material extrusion	Huang et al. (2013) and Wang et al. (2006)
Gel-MA		Material extrusion	Huang et al. (2013)
		Vat polymerization	Soman et al. (2013)
Fibrinogen and thrombin		Material extrusion	de Melo et al. (2019) and Skardal, Zhang, and Prestwich (2010)
		Material jetting	Albanna et al. (2019) and Gudapati, Parisi, Colby, and Ozbolat (2020)
Hyaluronan		Material extrusion	Skardal, Zhang, and Prestwich (2010)
Skeletal muscle dECM and vascular dECM		Material extrusion	Choi et al. (2019)
Liver dECM with GelMA		Vat polymerization	Ma et al. (2018)
Synthetic hydrogel			
PEG		Material jetting	Gao et al. (2014)
PEGDA		Acoustic droplet ejection	Fang et al. (2012)
		Material extrusion	Skardal, Zhang, and Prestwich (2010)
		Vat polymerization	Chan, Zorlutuna, Jeong, Kong, and Bashir (2010)
		Dynamic optical projection stereolithography (DOPsL)	Lu, Mapili, Suhali, Chen, and Roy (2006)
PLGA		Material extrusion	Naseri, Butler, MacNevin, Ahmed, and Ahmadi (2020)
PLGA-PEG		Material extrusion	Guo, Lim, Noshin, Ringel, and Fisher (2018) and Sawkins et al. (2012)
Pluronic F127		Material extrusion	Müller, Becher, Schnabelrauch, and Zenobi-Wong (2015)
Bioinspired hydrogel			
DNA-conjugated PLGA–PLG polymer		Material jetting	Li et al. (2015)
DNA-functionalized agarose mixed in gelatin and agarose		Material extrusion	Muller, Jakel, Schwarz, Aufinger, and Simmel (2020)
Hybrid			
Thermoplastic PCL, PLGA	Hydrogel HA, gelatin, collagen, fibrinogen	Melt-plotting system, material extrusion	Kang et al. (2016), Lee et al. (2013), Schuurman et al. (2011), and Shim et al. (2011)
PCL	Fibrinogen, collagen	Electrospinning, material jetting	Xu, Binder, et al. (2013)

noteworthy that advancement and innovation in areas revolving around material sciences and methods to process materials would advance the field of bioprinting.

References

- Ahmed, T. A., Dare, E. V., & Hincke, M. (2008). Fibrin: A versatile scaffold for tissue engineering applications. *Tissue Engineering. Part B, Reviews*, 14(2), 199–215. Available from <https://doi.org/10.1089/ten.teb.2007.0435>.
- Ahn, S., Lee, H., & Kim, G. (2013). Functional cell-laden alginate scaffolds consisting of core/shell struts for tissue regeneration. *Carbohydrate Polymers*, 98(1), 936–942. Available from <https://doi.org/10.1016/j.carbpol.2013.07.008>.
- Albanna, M., Binder, K. W., Murphy, S. V., Kim, J., Qasem, S. A., Zhao, W., . . . Marco, J. (2019). In situ bioprinting of autologous skin cells accelerates wound healing of extensive excisional full-thickness wounds. *Scientific Reports*, 9(1), 1–15.
- Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., & Watson, J. D. (1994). *Molecular biology of the cell* (3rd ed.). New York, NY: Garland Science.
- Alcantar, N. A., Aydil, E. S., & Israelachvili, J. N. (2000). Polyethylene glycol-coated biocompatible surfaces. *Journal of Biomedical Materials Research*, 51(3), 343–351.
- am Ende, M. T., Hariharan, D., & Peppas, N. A. (1995). Factors influencing drug and protein transport and release from ionic hydrogels. *Reactive Polymers*, 25(2–3), 127–137. Available from [http://doi.org/10.1016/0923-1137\(94\)00040-C](http://doi.org/10.1016/0923-1137(94)00040-C).
- Barbucci, R., Pasqui, D., Favalaro, R., & Panariello, G. (2008). A thixotropic hydrogel from chemically cross-linked guar gum: Synthesis, characterization and rheological behaviour. *Carbohydrate Research*, 343(18), 3058–3065. Available from <http://doi.org/10.1016/j.carres.2008.08.029>.
- Basu, A., Kunduru, K. R., Doppalapudi, S., Domb, A. J., & Khan, W. (2016). Poly(lactic acid) based hydrogels. *Advanced Drug Delivery Reviews*, 107, 192–205. Available from <https://doi.org/10.1016/j.addr.2016.07.004>.
- Bee, S.-L., Hamid, Z. A. A., Mariatti, M., Yahaya, B. H., Lim, K., Bee, S.-T., & Sin, L. T. (2018). Approaches to improve therapeutic efficacy of biodegradable PLA/PLGA microspheres: A review. *Polymer Reviews*, 58(3), 495–536. Available from <https://doi.org/10.1080/15583724.2018.1437547>.
- Belkas, J. S., Munro, C. A., Shoichet, M. S., Johnston, M., & Midha, R. (2005). Long-term in vivo biomechanical properties and biocompatibility of poly (2-hydroxyethyl methacrylate-co-methyl methacrylate) nerve conduits. *Biomaterials*, 26(14), 1741–1749.
- Bertlein, S., Brown, G., Lim, K. S., Jungst, T., Boeck, T., Blunk, T., . . . Groll, J. (2017). Thiol-ene clickable gelatin: A platform bioink for multiple 3D biofabrication technologies. *Advanced Materials*, 29(44), 1703404. Available from <https://doi.org/10.1002/adma.201703404>.
- Billiet, T., Gevaert, E., De Schryver, T., Cornelissen, M., & Dubruel, P. (2014). The 3D printing of gelatin methacrylamide cell-laden tissue-engineered constructs with high cell viability. *Biomaterials*, 35(1), 49–62. Available from <https://doi.org/10.1016/j.biomaterials.2013.09.078>.
- Brannon-Peppas, L., & Peppas, N. A. (1991). Equilibrium swelling behavior of pH-sensitive hydrogels. *Chemical Engineering Science*, 46(3), 715–722. Available from [http://doi.org/10.1016/0009-2509\(91\)80177-Z](http://doi.org/10.1016/0009-2509(91)80177-Z).
- Bryant, S. J., Nuttelman, C. R., & Anseth, K. S. (2000). Cytocompatibility of UV and visible light photoinitiating systems on cultured NIH/3T3 fibroblasts in vitro. *Journal of Biomaterials Science—Polymer Edition*, 11(5), 439–457. Available from <http://search.ebscohost.com/login.aspx?direct=true&db=aph&AN=4784400&site=ehost-live>.

- Chan, B. P., & Leong, K. W. (2008). Scaffolding in tissue engineering: General approaches and tissue-specific considerations. *European Spine Journal*, 17(4), 467–479. Available from <https://doi.org/10.1007/s00586-008-0745-3>.
- Chan, V., Zorlutuna, P., Jeong, J. H., Kong, H., & Bashir, R. (2010). Three-dimensional photopatterning of hydrogels using stereolithography for long-term cell encapsulation. *Lab on a Chip*, 10(16), 2062–2070. Available from <https://doi.org/10.1039/c004285d>.
- Chiang, C.-H., Fu, Y.-H., & Horng, J.-C. (2017). Formation of AAB-type collagen heterotrimers from designed cationic and aromatic collagen-mimetic peptides: Evaluation of the C-terminal cation – ational ctions. *Biomacromolecules*, 18(3), 985–993. Available from <https://doi.org/10.1021/acs.biomac.6b01838>.
- Choi, C., Kim, S., & Cha, C. (2021). Dual-functional alginate crosslinker: Independent control of crosslinking density and cell adhesive properties of hydrogels via separate conjugation pathways. *Carbohydrate Polymers*, 252, 117128. Available from <https://doi.org/10.1016/j.carbpol.2020.117128>.
- Choi, Y.-J., Jun, Y.-J., Kim, D. Y., Yi, H.-G., Chae, S.-H., Kang, J., ... Cho, D.-W. (2019). A 3D cell printed muscle construct with tissue-derived bioink for the treatment of volumetric muscle loss. *Biomaterials*, 206, 160–169. Available from <https://doi.org/10.1016/j.biomaterials.2019.03.036>.
- Chun, Y. S., Verma, K., Rosen, H., Lipsitz, S., Morris, D., Kenney, P., & Eriksson, E. (2010). Implant-based breast reconstruction using acellular dermal matrix and the risk of postoperative complications. *Plastic and Reconstructive Surgery*, 125(2), 429–436. Available from <https://doi.org/10.1097/PRS.0b013e3181c82d90>.
- Collins, M. C., & Ramirez, W. F. (1979). Transport through polymeric membranes. *The Journal of Physical Chemistry*, 83(17), 2294–2301. Available from <https://doi.org/10.1021/j100480a022>.
- Collins, M. N., & Birkinshaw, C. (2013). Hyaluronic acid based scaffolds for tissue engineering: A review. *Carbohydrate Polymers*, 92(2), 1262–1279. Available from <https://doi.org/10.1016/j.carbpol.2012.10.028>.
- Costantini, M., Testa, S., Mozetic, P., Barbetta, A., Fuoco, C., Fornetti, E., ... Świąszkowski, W. (2017). Microfluidic-enhanced 3D bioprinting of aligned myoblast-laden hydrogels leads to functionally organized myofibers in vitro and in vivo. *Biomaterials*, 131, 98–110.
- Crank, J. (1979). *The mathematics of diffusion*. Oxford University Press.
- Crank, J., & Park, G. S. (1968). *Diffusion in polymers*. Academic Press.
- Curley, J. L., Jennings, S. R., & Moore, M. J. (2011). Fabrication of micropatterned hydrogels for neural culture systems using dynamic mask projection photolithography. *JOVE-Journal of Visualized Experiments*, 48, 7. Available from <https://doi.org/10.3791/2636>.
- da Silva, L. P., Jha, A. K., Corrello, V. M., Marques, A. P., Reis, R. L., & Healy, K. E. (2018). Gellan Gum Hydrogels with Enzyme-Sensitive Biodegradation and Endothelial Cell Biorecognition Sites. *Advanced Healthcare Materials*, 7(5), 1700686. Available from <https://doi.org/10.1002/adhm.201700686>.
- Dare, E., Vascotto, S., Carlsson, D., Hincke, M., & Griffith, M. (2007). Differentiation of a fibrin gel encapsulated chondrogenic cell line. *The International Journal of Artificial Organs*, 30(7), 619.
- de Melo, B. A. G., Jodat, Y. A., Mehrotra, S., Calabrese, M. A., Kamperman, T., Mandal, B. B., ... Shin, S. R. (2019). 3D printed cartilage-like tissue constructs with spatially controlled mechanical properties. *Advanced Functional Materials*, 29(51), 1906330. Available from <https://doi.org/10.1002/adfm.201906330>.
- Dhandayuthapani, B., Yoshida, Y., Maekawa, T., & Sakthi Kumar, D. (2011). Polymeric scaffolds in tissue engineering application: A review. *International Journal of Polymer Science*, 2011. Available from <https://doi.org/10.1155/2011/290602>.
- Donati, I., & Paoletti, S. (2009). Material properties of alginates. *Alginates: Biology and Applications*, 13, 1–53. Available from https://doi.org/10.1007/978-3-540-92679-5_1.
- Dong, Y., Yao, C., Zhu, Y., Yang, L., Luo, D., & Yang, D. (2020). DNA functional materials assembled from branched DNA: Design, synthesis, and applications. *Chemical Reviews*, 120(17), 9420–9481. Available from <https://doi.org/10.1021/acs.chemrev.0c00294>.

- Drury, J. L., & Mooney, D. J. (2003). Hydrogels for tissue engineering: Scaffold design variables and applications. *Biomaterials*, 24(24), 4337–4351. Available from [https://doi.org/10.1016/S0142-9612\(03\)00340-5](https://doi.org/10.1016/S0142-9612(03)00340-5).
- Du, C., & Hill, R. J. (2019). Complementary-DNA-strand cross-linked polyacrylamide hydrogels. *Macromolecules*, 52(17), 6683–6697. Available from <https://doi.org/10.1021/acs.macromol.9b01338>.
- Du, Z., Xing, R., Cao, X., Yu, X., & Han, Y. (2017). Symmetric and uniform coalescence of ink-jetting printed polyfluorene ink drops by controlling the droplet spacing distance and ink surface tension/viscosity ratio. *Polymer*, 115, 45–51. Available from <https://doi.org/10.1016/j.polymer.2017.03.023>.
- Duan, B., Hockaday, L. A., Kang, K. H., & Butcher, J. T. (2013). 3D bioprinting of heterogeneous aortic valve conduits with alginate/gelatin hydrogels. *Journal of Biomedical Materials Research. Part A*, 101(5), 1255–1264. Available from <https://doi.org/10.1002/jbm.a.34420>.
- Duan, B., Kapetanovic, E., Hockaday, L. a, & Butcher, J. T. (2013). Three-dimensional printed trileaflet valve conduits using biological hydrogels and human valve interstitial cells. *Acta Biomaterialia*. Available from <https://doi.org/10.1016/j.actbio.2013.12.005>.
- Echalier, C., Jebors, S., Laconde, G., Brunel, L., Verdié, P., Causse, L., ... Subra, G. (2017). Sol–gel synthesis of collagen-inspired peptide hydrogel. *Materials Today*, 20(2), 59–66. Available from <https://doi.org/10.1016/j.mattod.2017.02.001>.
- Fang, Y., Frampton, J. P., Raghavan, S., Sabahi-Kaviani, R., Luker, G., Deng, C. X., & Takayama, S. (2012). Rapid generation of multiplexed cell cocultures using acoustic droplet ejection followed by aqueous two-phase exclusion patterning. *Tissue Engineering Part C: Methods*, 18(9), 647–657. Available from <https://doi.org/10.1089/ten.tec.2011.0709>.
- Faulkner-Jones, A., Fyfe, C., Cornelissen, D. J., Gardner, J., King, J., Courtney, A., & Shu, W. M. (2015). Bioprinting of human pluripotent stem cells and their directed differentiation into hepatocyte-like cells for the generation of mini-livers in 3D. *Biofabrication*, 7(4), 044102. Available from <https://doi.org/10.1088/1758-5090/7/4/044102>.
- Fedorovich, N. E., Schuurman, W., Wijnberg, H. M., Prins, H.-J., van Weeren, P. R., Malda, J., & Dhert, W. J. A. (2011). Biofabrication of osteochondral tissue equivalents by printing topologically defined, cell-laden hydrogel scaffolds. *Tissue Engineering*, 18(1), 33–44. Available from <https://doi.org/10.1089/ten.tec.2011.0060>.
- Flory, P. J., & Rehner, J. (1943). Statistical mechanics of cross-linked polymer networks II. Swelling. *The Journal of Chemical Physics*, 11(11), 521–526. Available from <http://doi.org/10.1063/1.1723792>.
- Gao, G., Schilling, A. F., Yonezawa, T., Wang, J., Dai, G., & Cui, X. (2014). Bioactive nanoparticles stimulate bone tissue formation in bioprinted three-dimensional scaffold and human mesenchymal stem cells. *Biotechnology Journal*, 9(10), 1304–1311. Available from <https://doi.org/10.1002/biot.201400305>.
- Gao, Q., He, Y., Fu, J.-z, Liu, A., & Ma, L. (2015). Coaxial nozzle-assisted 3D bioprinting with built-in micro-channels for nutrients delivery. *Biomaterials*, 61, 203–215. Available from <http://doi.org/10.1016/j.biomaterials.2015.05.031>.
- Gudapati, H., Parisi, D., Colby, R. H., & Ozbolat, I. T. (2020). Rheological investigation of collagen, fibrinogen, and thrombin solutions for drop-on-demand 3D bioprinting. *Soft Matter*, 16(46), 10506–10517. Available from <https://doi.org/10.1039/D0SM01455A>.
- Gudeman, L. F., & Peppas, N. A. (1995). pH-sensitive membranes from poly(vinyl alcohol)/poly(acrylic acid) interpenetrating networks. *Journal of Membrane Science*, 107(3), 239–248. Available from [http://doi.org/10.1016/0376-7388\(95\)00120-7](http://doi.org/10.1016/0376-7388(95)00120-7).
- Guo, T., Lim, C. G., Noshin, M., Ringel, J. P., & Fisher, J. P. (2018). 3D printing bioactive PLGA scaffolds using DMSO as a removable solvent. *Bioprinting*, 10, e00038. Available from <https://doi.org/10.1016/j.bprint.2018.e00038>.
- Han, D., Lu, Z., Chester, S. A., & Lee, H. (2018). Micro 3D printing of a temperature-responsive hydrogel using projection micro-stereolithography. *Scientific Reports*, 8(1), 1963. Available from <https://doi.org/10.1038/s41598-018-20385-2>.

- He, Z., Zang, H., Zhu, L., Huang, K., Yi, T., Zhang, S., & Cheng, S. (2019). An anti-inflammatory peptide and brain-derived neurotrophic factor-modified hyaluronan-methylcellulose hydrogel promotes nerve regeneration in rats with spinal cord injury. *International Journal of Nanomedicine*, 14, 721–732. Available from <https://doi.org/10.2147/ijn.S187854>.
- Hinton, T. J., Jallerat, Q., Palchesko, R. N., Park, J. H., Grodzicki, M. S., Shue, H.-J., ... Feinberg, A. W. (2015). Three-dimensional printing of complex biological structures by freeform reversible embedding of suspended hydrogels. *Science Advances*, 1(9), e1500758. Available from <https://doi.org/10.1126/sciadv.1500758>.
- Hoffman, A. S. (2002). Hydrogels for biomedical applications. *Advanced Drug Delivery Reviews*, 54(1), 3–12. Available from [http://doi.org/10.1016/S0169-409X\(01\)00239-3](http://doi.org/10.1016/S0169-409X(01)00239-3).
- Hosoyama, K., Lazurko, C., Muñoz, M., McTiernan, C. D., & Alarcon, E. I. (2019). Peptide-based functional biomaterials for soft-tissue repair. *Frontiers in Bioengineering and Biotechnology*, 7(205). Available from <https://doi.org/10.3389/fbioe.2019.00205>.
- Huang, Y., He, K., & Wang, X. (2013). Rapid prototyping of a hybrid hierarchical polyurethane-cell/hydrogel construct for regenerative medicine. *Materials Science & Engineering. C, Materials for Biological Applications*, 33(6), 3220–3229. Available from <https://doi.org/10.1016/j.msec.2013.03.048>.
- Huettner, N., Dargaville, T. R., & Forget, A. (2018). Discovering cell-adhesion peptides in tissue engineering: Beyond RGD. *Trends in Biotechnology*, 36(4), 372–383. Available from <https://doi.org/10.1016/j.tibtech.2018.01.008>.
- Huglin, M. B., & Yip, D. C. F. (1987). An alternative method of determining the water content of hydrogels. *Die Makromolekulare Chemie, Rapid Communications*, 8(5), 237–242. Available from <https://doi.org/10.1002/marc.1987.030080506>.
- Hull, S. M., Lindsay, C. D., Brunel, L. G., Shiawski, D. J., Tashman, J. W., Roth, J. G., ... Heilshorn, S. C. (2020). 3D bioprinting using UNiVersal Orthogonal Network (UNION) bioinks. *Advanced Functional Materials*, 2007983. Available from <https://doi.org/10.1002/adfm.202007983>.
- Irvine, S. A., Agrawal, A., Lee, B. H., Chua, H. Y., Low, K. Y., Lau, B. C., ... Venkatraman, S. (2015). Printing cell-laden gelatin constructs by free-form fabrication and enzymatic protein crosslinking. *Biomedical Microdevices*, 17(1), 16. Available from <https://doi.org/10.1007/s10544-014-9915-8>.
- Jang, D., Kim, D., & Moon, J. (2009). Influence of fluid physical properties on ink-jet printability. *Langmuir: The ACS Journal of Surfaces and Colloids*, 25(5), 2629–2635. Available from <https://doi.org/10.1021/la900059m>.
- Johnson, F. A., Craig, D. Q., & Mercer, A. D. (1997). Characterization of the block structure and molecular weight of sodium alginates. *Journal of Pharmacy and Pharmacology*, 49(7), 639–643.
- Kang, H.-W., Lee, S. J., Ko, I. K., Kengla, C., Yoo, J. J., & Atala, A. (2016). A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. *Nature Biotechnology*, 34(3), 312–319. Available from <https://doi.org/10.1038/nbt.3413>.
- Kang, K., Hockaday, L., & Butcher, J. (2013). Quantitative optimization of solid freeform deposition of aqueous hydrogels. *Biofabrication*, 5(3), 035001.
- Kesti, M., Eberhardt, C., Pagliccia, G., Kenkel, D., Grande, D., Boss, A., & Zenobi-Wong, M. (2015). Bioprinting complex cartilaginous structures with clinically compliant biomaterials. *Advanced Functional Materials*, 25(48), 7406–7417. Available from <https://doi.org/10.1002/adfm.201503423>.
- Kesti, M., & Zenobi-Wong, M. (2015). Bioprinting complex cartilaginous structures with ECM-based bioinks. *Tissue Engineering. Part A*, 21, S285. Retrieved from <Go to ISI>://WOS:000360205202208.
- Khan, F., & Ahmad, S. R. (2013). Polysaccharides and their derivatives for versatile tissue engineering application. *Macromolecular Bioscience*, 13(4), 395–421. Available from <https://doi.org/10.1002/mabi.201200409>.
- Khoo, S. L., Riccardo, L., Pedro, F. C., Miguel, D. C., Cesar, R. A.-O., Kim, M. A. v D., ... Tim, B. F. W. (2018). Bio-resin for high resolution lithography-based biofabrication of complex cell-laden constructs. *Biofabrication*, 10(3), 034101. Available from <http://stacks.iop.org/1758-5090/10/i=3/a=034101>.

- Khor, E., & Lim, L. Y. (2003). Implantable applications of chitin and chitosan. *Biomaterials*, 24(13), 2339–2349.
- Kim, Y. B., Lee, H., & Kim, G. H. (2016). Strategy to achieve highly porous/biocompatible macroscale cell blocks, using a collagen/genipin-bioink and an optimal 3D printing process. *ACS Applied Materials & Interfaces*, 8(47), 32230–32240.
- Klouda, L., Hacker, M. C., Kretlow, J. D., & Mikos, A. G. (2009). Cytocompatibility evaluation of amphiphilic, thermally responsive and chemically crosslinkable macromers for *in situ* forming hydrogels. *Biomaterials*, 30(27), 4558–4566.
- Kolesky, D. B., Truby, R. L., Gladman, A. S., Busbee, T. A., Homan, K. A., & Lewis, J. A. (2014). 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. *Advanced Materials*, 26(19), 3124–3130. Available from <https://doi.org/10.1002/adma.201305506>.
- Lee, C., Grodzinsky, A., & Spector, M. (2001). The effects of cross-linking of collagen-glycosaminoglycan scaffolds on compressive stiffness, chondrocyte-mediated contraction, proliferation and biosynthesis. *Biomaterials*, 22(23), 3145–3154.
- Lee, C. H., Singla, A., & Lee, Y. (2001). Biomedical applications of collagen. *International Journal of Pharmaceutics*, 221(1), 1–22.
- Lee, H., Ahn, S., Bonassar, L. J., Chun, W., & Kim, G. (2013). Cell-laden poly(varepsilon-caprolactone)/alginate hybrid scaffolds fabricated by an aerosol cross-linking process for obtaining homogeneous cell distribution: Fabrication, seeding efficiency, and cell proliferation and distribution. *Tissue Engineering. Part C, Methods*, 19(10), 784–793. Available from <https://doi.org/10.1089/ten.TEC.2012.0651>.
- Lee, J. M., Sing, S. L., Zhou, M., & Yeong, W. Y. (2018). 3D bioprinting processes: A perspective on classification and terminology. *International Journal of Bioprinting*, 4(2). Available from <https://doi.org/10.18063/ijb.v4i2.151>.
- Lee, J.M. & Yeong, W.Y. (2014). Understanding the relation between printing and material parameters of time-pressure dispensing system for bioprinting. In *Proceedings of the first international conference on progress in additive manufacturing*.
- Lee, J. M., & Yeong, W. Y. (2015). A preliminary model of time-pressure dispensing system for bioprinting based on printing and material parameters. *Virtual and Physical Prototyping*, 10(1), 3–8. Available from <https://doi.org/10.1080/17452759.2014.979557>.
- Lee, J. M., & Yeong, W. Y. (2016). Design and printing strategies in 3D bioprinting of cell-hydrogels: A review. *Advanced Healthcare Materials*, 5(22), 2856–2865.
- Lee, J. M., & Yeong, W. Y. (2020). Engineering macroscale cell alignment through coordinated toolpath design using support-assisted 3D bioprinting. *Journal of the Royal Society Interface*, 17(168), 20200294. Available from <https://doi.org/10.1098/rsif.2020.0294>.
- Lee, K. Y., Bouhadir, K. H., & Mooney, D. J. (2004). Controlled degradation of hydrogels using multifunctional cross-linking molecules. *Biomaterials*, 25(13), 2461–2466. Available from <http://doi.org/10.1016/j.biomaterials.2003.09.030>.
- Lee, M. P., Cooper, G. J. T., Hinkley, T., Gibson, G. M., Padgett, M. J., & Cronin, L. (2015). Development of a 3D printer using scanning projection stereolithography. *Scientific Reports*, 5, 9875. Available from <https://doi.org/10.1038/srep09875>.
- Lee, W., Lee, V. K., & Polio, S. (2009). Three-dimensional cell-hydrogel printer using electromechanical microvalve for tissue engineering. *Solid-State Sensors*, 2230–2233. Available from http://ieeexplore.ieee.org/xpls/abs_all.jsp?arnumber=5285591.
- Lee, Y. B., Polio, S., Lee, W., Dai, G., Menon, L., Carroll, R. S., & Yoo, S. S. (2010). Bio-printing of collagen and VEGF-releasing fibrin gel scaffolds for neural stem cell culture. *Experimental Neurology*, 223(2), 645–652. Available from <https://doi.org/10.1016/j.expneurol.2010.02.014>.

- Lei, Y., Ng, Q. K., & Segura, T. (2010). Two and three-dimensional gene transfer from enzymatically degradable hydrogel scaffolds. *Microscopy Research and Technique*, 73(9), 910–917.
- Li, C., Faulkner-Jones, A., Dun, A. R., Jin, J., Chen, P., Xing, Y., ... Duncan, R. R. (2015). Rapid formation of a supramolecular polypeptide–DNA hydrogel for in situ three-dimensional multilayer bioprinting. *Angewandte Chemie International Edition*, 54(13), 3957–3961. Available from <https://doi.org/10.1002/anie.201411383>.
- Li, F., Tang, J., Geng, J., Luo, D., & Yang, D. (2019). Polymeric DNA hydrogel: Design, synthesis and applications. *Progress in Polymer Science*, 98, 101163. Available from <https://doi.org/10.1016/j.progpolymsci.2019.101163>.
- Li, M. G., Tian, X. Y., & Chen, X. B. (2009). Modeling of flow rate, pore size, and porosity for the dispensing-based tissue scaffolds fabrication. *Journal of Manufacturing Science and Engineering*, 131(3), 034501. Available from <https://doi.org/10.1115/1.3123331>.
- Lu, Y., Mapili, G., Suhali, G., Chen, S., & Roy, K. (2006). A digital micro-mirror device-based system for the microfabrication of complex, spatially patterned tissue engineering scaffolds. *Journal of Biomedical Materials Research. Part A*, 77(2), 396–405. Available from <https://doi.org/10.1002/jbm.a.30601>.
- Lukomski, S., & McNitt, D. H. (2020). Expression and purification of collagen-like proteins of group A streptococcus. In T. Proft, & J. M. S. Loh (Eds.), *Group A streptococcus: Methods and protocols* (pp. 163–179). New York, NY: Springer US.
- Luo, Y., Dolder, C. K., Walker, J. M., Mishra, R., Dean, D., & Becker, M. L. (2016). Synthesis and biological evaluation of well-defined poly(propylene fumarate) oligomers and their use in 3D printed scaffolds. *Biomacromolecules*, 17(2), 690–697. Available from <https://doi.org/10.1021/acs.biomac.6b00014>.
- Ma, X. Y., Yu, C., Wang, P. R., Xu, W. Z., Wan, X. Y., Lai, C. S. E., ... Chen, S. C. (2018). Rapid 3D bioprinting of decellularized extracellular matrix with regionally varied mechanical properties and biomimetic microarchitecture. *Biomaterials*, 185, 310–321. Available from <https://doi.org/10.1016/j.biomaterials.2018.09.026>.
- Malda, J., Visser, J., Melchels, F. P., Jüngst, T., Hennink, W. E., Dhert, W. J. A., ... Huttmacher, D. W. (2013). 25th anniversary article: Engineering hydrogels for biofabrication. *Advanced Materials*, 25(36), 5011–5028. Available from <https://doi.org/10.1002/adma.201302042>.
- Mao, T., Kuhn, D., & Tran, H. (1997). Spread and rebound of liquid droplets upon impact on flat surfaces. *AIChE Journal*, 43(9), 2169–2179.
- Markstedt, K., Mantas, A., Tournier, I., Martínez Ávila, H., Hägg, D., & Gatenholm, P. (2015). 3D bioprinting human chondrocytes with nanocellulose–alginate bioink for cartilage tissue engineering applications. *Biomacromolecules*, 16(5), 1489–1496. Available from <https://doi.org/10.1021/acs.biomac.5b00188>.
- Miller, J. S., Stevens, K. R., Yang, M. T., Baker, B. M., Nguyen, D.-H. T., Cohen, D. M., ... Chen, C. S. (2012). Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues. *Nature Materials*, 11(9), 768–774. Available from <http://www.nature.com/nmat/journal/v11/n9/abs/nmat3357.html#supplementary-information>.
- Muller, J., Jakel, A. C., Schwarz, D., Aufinger, L., & Simmel, F. C. (2020). Programming diffusion and localization of DNA signals in 3D-printed DNA-functionalized hydrogels. *Small (Weinheim an der Bergstrasse, Germany)*, 16(31), 10. Available from <https://doi.org/10.1002/sml.202001815>.
- Müller, M., Becher, J., Schnabelrauch, M., & Zenobi-Wong, M. (2015). Nanostructured Pluronic hydrogels as bioinks for 3D bioprinting. *Biofabrication*, 7(3), 035006. Available from <https://doi.org/10.1088/1758-5090/7/3/035006>.
- Munim, S. A., & Raza, Z. A. (2019). Poly(lactic acid) based hydrogels: Formation, characteristics and biomedical applications. *Journal of Porous Materials*, 26(3), 881–901. Available from <https://doi.org/10.1007/s10934-018-0687-z>.

- Murphy, S. V., Skardal, A., & Atala, A. (2013). Evaluation of hydrogels for bio-printing applications. *Journal of Biomedical Materials Research. Part A*, 101A(1), 272–284. Available from <https://doi.org/10.1002/jbm.a.34326>.
- Nagaoka, S., Tanzawa, H., & Suzuki, J. (1990). Cell proliferation on hydrogels. *In Vitro Cellular & Developmental Biology*, 26(1), 51–56. Available from <https://doi.org/10.1007/BF02624154>.
- Naseri, E., Butler, H., MacNevin, W., Ahmed, M., & Ahmadi, A. (2020). Low-temperature solvent-based 3D printing of PLGA: A parametric printability study. *Drug Development and Industrial Pharmacy*, 46(2), 173–178. Available from <https://doi.org/10.1080/03639045.2019.1711389>.
- Ng, W. L., Lee, J. M., Yeong, W. Y., & Win Naing, M. (2017). Microvalve-based bioprinting—Process, bioinks and applications. *Biomaterials Science*, 5(4), 632–647.
- Nicodemus, G. D., & Bryant, S. J. (2008). Cell encapsulation in biodegradable hydrogels for tissue engineering applications. *Tissue Engineering Part B: Reviews*, 14(2), 149–165. Available from <http://online.liebertpub.com/doi/abs/10.1089/ten.teb.2007.0332>.
- Nishiyama, Y., Nakamura, M., Henmi, C., Yamaguchi, K., Mochizuki, S., Nakagawa, H., & Takiura, K. (2008). Development of a three-dimensional bioprinter: Construction of cell supporting structures using hydrogel and state-of-the-art inkjet technology. *Journal of Biomechanical Engineering*, 131(3), 035001. Available from <https://doi.org/10.1115/1.3002759>.
- Ogawa, T., Akazawa, T., & Tabata, Y. (2010). In vitro proliferation and chondrogenic differentiation of rat bone marrow stem cells cultured with gelatin hydrogel microspheres for TGF- β 1 release. *Journal of Biomaterials Science—Polymer Edition*, 21(5), 609–621. Available from <https://doi.org/10.1163/156856209X434638>.
- Ozbolat, I. T., Chen, H., & Yu, Y. (2014). Development of ‘Multi-arm Bioprinter’ for hybrid biofabrication of tissue engineering constructs. *Robotics and Computer-Integrated Manufacturing*, 30(3), 295–304. Available from <https://doi.org/10.1016/j.rcim.2013.10.005>.
- Pal, V. K., Jain, R., & Roy, S. (2020). Tuning the supramolecular structure and function of collagen mimetic ionic complementary peptides via electrostatic interactions. *Langmuir: The ACS Journal of Surfaces and Colloids*, 36(4), 1003–1013. Available from <https://doi.org/10.1021/acs.langmuir.9b02941>.
- Panwar, A., & Tan, L. P. (2016). Current status of bioinks for micro-extrusion-based 3D bioprinting. *Molecules (Basel, Switzerland)*, 21(6), 685. Available from <https://doi.org/10.3390/molecules21060685>.
- Park, K. M., Lee, S. Y., Joong, Y. K., Na, J. S., Lee, M. C., & Park, K. D. (2009). Thermosensitive chitosan–Pluronic hydrogel as an injectable cell delivery carrier for cartilage regeneration. *Acta Biomaterialia*, 5(6), 1956–1965. Available from <http://doi.org/10.1016/j.actbio.2009.01.040>.
- Park, S.-N., Park, J.-C., Kim, H. O., Song, M. J., & Suh, H. (2002). Characterization of porous collagen/hyaluronic acid scaffold modified by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide cross-linking. *Biomaterials*, 23(4), 1205–1212.
- Pati, F., Jang, J., Ha, D.-H., Won Kim, S., Rhie, J.-W., Shim, J.-H., ... Cho, D.-W. (2014). Printing three-dimensional tissue analogues with decellularized extracellular matrix bioink. *Nature Communications*, 5. Available from <https://doi.org/10.1038/ncomms4935>.
- Peppas, N. A., Hilt, J. Z., Khademhosseini, A., & Langer, R. (2006). Hydrogels in biology and medicine: From molecular principles to bionanotechnology. *Advanced Materials*, 18(11), 1345–1360. Available from <https://doi.org/10.1002/adma.200501612>.
- Peppas, N. A., & Merrill, E. W. (1977). Crosslinked poly(vinyl alcohol) hydrogels as swollen elastic networks. *Journal of Applied Polymer Science*, 21(7), 1763–1770. Available from <https://doi.org/10.1002/app.1977.070210704>.
- Peppas, N. A., & Reinhart, C. T. (1983). Solute diffusion in swollen membranes. Part I. A new theory. *Journal of Membrane Science*, 15(3), 275–287. Available from [http://doi.org/10.1016/S0376-7388\(00\)82304-2](http://doi.org/10.1016/S0376-7388(00)82304-2).
- Puthia, M., Butrym, M., Petrlova, J., Strömdahl, A.-C., Andersson, M. Å., Kjellström, S., & Schmidtchen, A. (2020). A dual-action peptide-containing hydrogel targets wound infection and inflammation. *Science Translational Medicine*, 12(524). Available from <https://doi.org/10.1126/scitranslmed.aax6601>, eaax6601.

- Ratner, B. D., Hoffman, A. S., Schoen, F. J., & Lemons, J. E. (2013). *Biomaterials science: an introduction to materials in medicine* (3rd ed.). London, UK: Elsevier.
- Ren, D. W., Yi, H. F., Wang, W., & Ma, X. J. (2005). The enzymatic degradation and swelling properties of chitosan matrices with different degrees of N-acetylation. *Carbohydrate Research*, 340(15), 2403–2410. Available from <https://doi.org/10.1016/j.carres.2005.07.022>.
- Rhee, S., Puetzer, J. L., Mason, B. N., Reinhart-King, C. A., & Bonassar, L. J. (2016). 3D bioprinting of spatially heterogeneous collagen constructs for cartilage tissue engineering. *ACS Biomaterials Science & Engineering*, 2(10), 1800–1805.
- Richardson, T., Wiegand, C., Adisa, F., Ravikumar, K., Candiello, J., Kumta, P., & Banerjee, I. (2020). Engineered peptide modified hydrogel platform for propagation of human pluripotent stem cells. *Acta Biomaterialia*, 113, 228–239. Available from <https://doi.org/10.1016/j.actbio.2020.06.034>.
- Sakai, S., Kamei, H., Mori, T., Hotta, T., Ohi, H., Nakahata, M., & Taya, M. (2018). Visible light-induced hydrogelation of an alginate derivative and application to stereolithographic bioprinting using a visible light projector and acid red. *Biomacromolecules*, 19(2), 672–679. Available from <https://doi.org/10.1021/acs.biomac.7b01827>.
- Sawkins, M.J., Brown, B.N., Bonassar, L.J., Rose, F., & Shakesheff, K.M. (2012). *Bioplotting of novel scaffold materials and complex constructs for osteochondral tissue engineering*. 23, 2262.
- Schmedlen, R. H., Masters, K. S., & West, J. L. (2002). Photocrosslinkable polyvinyl alcohol hydrogels that can be modified with cell adhesion peptides for use in tissue engineering. *Biomaterials*, 23(22), 4325–4332.
- Schuurman, W., Khristov, V., Pot, M. W., van Weeren, P. R., Dhert, W. J., & Malda, J. (2011). Bioprinting of hybrid tissue constructs with tailorable mechanical properties. *Biofabrication*, 3(2), 021001. Available from <https://doi.org/10.1088/1758-5082/3/2/021001>.
- Schuurman, W., Levett, P. A., Pot, M. W., van Weeren, P. R., Dhert, W. J. A., Hutmacher, D. W., ... Malda, J. (2013). Gelatin-methacrylamide hydrogels as potential biomaterials for fabrication of tissue-engineered cartilage constructs. *Macromolecular Bioscience*, 13(5), 551–561. Available from <https://doi.org/10.1002/mabi.201200471>.
- Seo, Y., Jung, Y., & Kim, S. H. (2018). Decellularized heart ECM hydrogel using supercritical carbon dioxide for improved angiogenesis. *Acta Biomaterialia*, 67, 270–281. Available from <https://doi.org/10.1016/j.actbio.2017.11.046>.
- Shim, J. H., Kim, J. Y., Park, M., Park, J., & Cho, D. W. (2011). Development of a hybrid scaffold with synthetic biomaterials and hydrogel using solid freeform fabrication technology. *Biofabrication*, 3(3), 034102. Available from <https://doi.org/10.1088/1758-5082/3/3/034102>.
- Shim, J.-H., Lee, J.-S., Kim, J. Y., & Cho, D.-W. (2012). Bioprinting of a mechanically enhanced three-dimensional dual cell-laden construct for osteochondral tissue engineering using a multi-head tissue/organ building system. *Journal of Micromechanics and Microengineering*, 22(8), 085014. Available from <https://doi.org/10.1088/0960-1317/22/8/085014>.
- Skardal, A., Mack, D., Kapetanovic, E., Atala, A., Jackson, J. D., Yoo, J., & Soker, S. (2012). Bioprinted amniotic fluid-derived stem cells accelerate healing of large skin wounds. *Stem Cells Translational Medicine*, 1(11), 792–802. Available from <https://doi.org/10.5966/sctm.2012-0088>.
- Skardal, A., Zhang, J., McCoard, L., Xu, X., Oottamasathien, S., & Prestwich, G. D. (2010). Photocrosslinkable hyaluronan-gelatin hydrogels for two-step bioprinting. *Tissue Engineering. Part A*, 16(8), 2675–2685. Available from <https://doi.org/10.1089/ten.tea.2009.0798>.
- Skardal, A., Zhang, J., & Prestwich, G. D. (2010). Bioprinting vessel-like constructs using hyaluronan hydrogels crosslinked with tetrahedral polyethylene glycol tetracrylates. *Biomaterials*, 31(24), 6173–6181. Available from <https://doi.org/10.1016/j.biomaterials.2010.04.045>.
- Slaughter, B. V., Khurshid, S. S., Fisher, O. Z., Khademhosseini, A., & Peppas, N. A. (2009). Hydrogels in regenerative medicine. *Advanced Materials*, 21(32-33), 3307–3329.

- Snyder, J. E., Hamid, Q., Wang, C., Chang, R., Emami, K., Wu, H., & Sun, W. (2011). Bioprinting cell-laden matrigel for radioprotection study of liver by pro-drug conversion in a dual-tissue microfluidic chip. *Biofabrication*, 3(3), 034112. Available from <https://doi.org/10.1088/1758-5082/3/3/034112>.
- Soman, P., Chung, P. H., Zhang, aP., & Chen, S. (2013). Digital microfabrication of user-defined 3D microstructures in cell-laden hydrogels. *Biotechnology and Bioengineering*, 110(11), 3038–3047. Available from <https://doi.org/10.1002/bit.24957>.
- Su, J., Satchell, S. C., Wertheim, J. A., & Shah, R. N. (2019). Poly(ethylene glycol)-crosslinked gelatin hydrogel substrates with conjugated bioactive peptides influence endothelial cell behavior. *Biomaterials*, 201, 99–112. Available from <https://doi.org/10.1016/j.biomaterials.2019.02.001>.
- Sydney Gladman, A., Matsumoto, E. A., Nuzzo, R. G., Mahadevan, L., & Lewis, J. A. (2016). Biomimetic 4D printing. *Nature Materials*, 15(4), 413–418. Available from <https://doi.org/10.1038/nmat4544>.
- Tan, E. Y. S., & Yeong, W. Y. (2015). Concentric bioprinting of alginate-based tubular constructs using multi-nozzle extrusion-based technique. *International Journal of Bioprinting*, 1(1), 49–56. Available from <http://doi.org/10.18063/IJB.2015.01.003>.
- Tan, Y. S. E., & Yeong, W. Y. (2014). Direct bioprinting of alginate-based tubular constructs using multi-nozzle extrusion-based technique. In *Proceedings of the first international conference on progress in additive manufacturing*.
- Taylor, D. A., Sampaio, L. C., Ferdous, Z., Gobin, A. S., & Taite, L. J. (2018). Decellularized matrices in regenerative medicine. *Acta Biomaterialia*, 74, 74–89. Available from <https://doi.org/10.1016/j.actbio.2018.04.044>.
- Tesoro, G. (1984). Textbook of polymer science, 3rd ed., Fred W. Billmeyer, Jr., Wiley-Interscience, New York, 1984, 578 pp. No price given. *Journal of Polymer Science: Polymer Letters Edition*, 22(12), 674. Available from <https://doi.org/10.1002/pol.1984.130221210>.
- Visser, J., Peters, B., Burger, T. J., Boomstra, J., Dhert, W. J. a, Melchels, F. P. W., & Malda, J. (2013). Biofabrication of multi-material anatomically shaped tissue constructs. *Biofabrication*, 5(3), 035007. Available from <https://doi.org/10.1088/1758-5082/5/3/035007>.
- Wang, X., Yan, Y., Pan, Y., Xiong, Z., Liu, H., Cheng, J., . . . Lu, Q. (2006). Generation of three-dimensional hepatocyte/gelatin structures with rapid prototyping system. *Tissue Engineering*, 12(1), 83–90. Available from <https://doi.org/10.1089/ten.2006.12.83>.
- Wang, Z., Jin, X., Tian, Z., Menard, F., Holzman, J. F., & Kim, K. (2018). A novel, well-resolved direct laser bioprinting system for rapid cell encapsulation and microwell fabrication. *Advanced Healthcare Materials*, 7(9). Available from <https://doi.org/10.1002/adhm.201701249>.
- Wu, W., DeConinck, A., & Lewis, J. A. (2011). Omnidirectional printing of 3D microvascular networks. *Advanced Materials*, 23(24), H178. Available from <https://doi.org/10.1002/adma.201004625>.
- Wüst, S., Godla, M. E., Müller, R., & Hofmann, S. (2014). Tunable hydrogel composite with two-step processing in combination with innovative hardware upgrade for cell-based three-dimensional bioprinting. *Acta Biomaterialia*, 10(2), 630–640. Available from <http://doi.org/10.1016/j.actbio.2013.10.016>.
- Xing, Y., Cheng, E., Yang, Y., Chen, P., Zhang, T., Sun, Y., . . . Liu, D. (2011). Self-assembled DNA hydrogels with designable thermal and enzymatic responsiveness. *Advanced Materials*, 23(9), 1117–1121. Available from <https://doi.org/10.1002/adma.201003343>.
- Xu, C., Chai, W., Huang, Y., & Markwald, R. R. (2012). Scaffold-free inkjet printing of three-dimensional zig-zag cellular tubes. *Biotechnology and Bioengineering*, 109(12), 3152–3160. Available from <https://doi.org/10.1002/bit.24591>.
- Xu, C., Zhang, Z., Fu, J., & Huang, Y. (2017). Study of pinch-off locations during drop-on-demand inkjet printing of viscoelastic alginate solutions. *Langmuir: The ACS Journal of Surfaces and Colloids*, 33(20), 5037–5045. Available from <https://doi.org/10.1021/acs.langmuir.7b00874>.

- Xu, T., Binder, K. W., Albanna, M. Z., Dice, D., Zhao, W., Yoo, J. J., & Atala, A. (2013). Hybrid printing of mechanically and biologically improved constructs for cartilage tissue engineering applications. *Biofabrication*, 5(1), 015001. Available from <https://doi.org/10.1088/1758-5082/5/1/015001>.
- Xu, T., Zhao, W., Zhu, J. M., Albanna, M. Z., Yoo, J. J., & Atala, A. (2013). Complex heterogeneous tissue constructs containing multiple cell types prepared by inkjet printing technology. *Biomaterials*, 34(1), 130–139. Available from <https://doi.org/10.1016/j.biomaterials.2012.09.035>.
- Yucel, T., Cebe, P., & Kaplan, D. L. (2009). Vortex-induced injectable silk fibroin hydrogels. *Biophysical Journal*, 97(7), 2044–2050. Available from <http://doi.org/10.1016/j.bpj.2009.07.028>.
- Zhao, Z., Wang, C., Yan, H., & Liu, Y. (2019). Soft robotics programmed with double crosslinking DNA hydrogels. *Advanced Functional Materials*, 29(45), 1905911. Available from <https://doi.org/10.1002/adfm.201905911>.

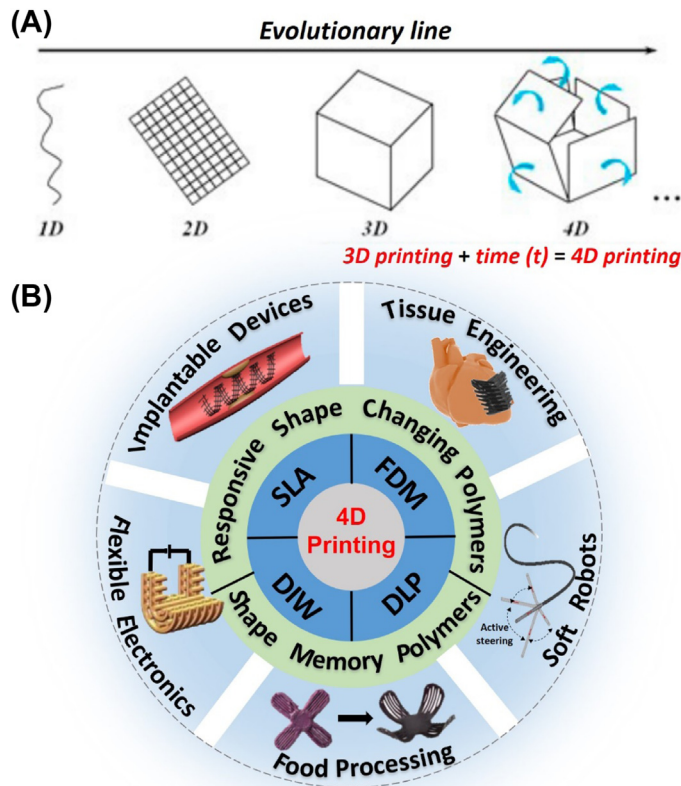
4D Printing: 3D Printing of Responsive and Programmable Materials

Heng Deng and Jian Lin

Department of Mechanical and Aerospace Engineering, University of Missouri, Columbia, MO, United States

8.1 INTRODUCTION

The idea of fabricating architectures from programmable materials was first conceptualized in 2012 (Tibbitts, 2012; Tibbitts & Cheung, 2012). The concept was later advanced to four-dimensional (4D) printing (Ge, Qi, & Dunn, 2013; Tibbitts, 2014). The concept of 4D printing was expressed as “3D printing + time” (Figure 8.1A), which refers to a targeted shape evolution of 3D-printed objects over time (Leist & Zhou, 2016; Momeni, Mehdi, Hassani, Liu, & Ni, 2017; Quanjin et al., 2020). Since then, unprecedented interests have been attracted to this emerging field. With the number of studies in 4D printing increasing, the concept of 4D printing has also evolved in the past few years. A more comprehensive definition of 4D printing was brought forward: 3D-printed structures can adaptively change their shapes, properties, and functions over time when exposed to predetermined stimuli (such as water, light, heat, electricity, and pH) (Chu et al., 2020). The traditional 3D-printing technique is about printing the finished product through premodeling, while the idea of 4D printing is to integrate the design of the product into a stimulus-responsive material based on 3D printing. As a result, the product produced by 4D printing can change their shapes according to the programmed track under specific activation conditions on demand. In a typical 4D printing process, stimulus-responsive active materials with various responses to external stimuli such as swelling or thermal expansion are printed via 3D-printing facilities according to their designed shape-transformation behaviors (Shafranek et al., 2019). Compared to traditional 3D printing, 4D printing is advantageous in terms of adaptability, thus opening new possibilities for applications in structures that can be activated for self-assembly, reconfiguration, and replication through environmental free energies (Momeni et al., 2017). This brings several advantages, such as significant volume reduction for storage, and transformations that can be achieved with flat-packed and 4D-printed structures (Tibbitts, 2014). Another example is that instead of directly creating a complex structure, simple components from smart materials can be 3D printed first and then self-assembled to reach preprogrammed complex shapes (Zhou et al., 2015). This is especially useful if multiple components made from different types of materials (e.g., polymer and inorganic semiconductors) must be integrated into one printed structure. Due to these technological advantages, 4D printing has been widely explored for application in a wide variety of fields (Figure 8.1B) such as biomedicine, soft robotics, flexible electronics, and food processing (Kuang et al., 2019).

**FIGURE 8.1**

Overview of 4D printing and their applications.

Adapted from Cui et al. (2020); Qianjin et al. (2020); Wan, Zhang, Liu, & Leng (2019); He et al. (2020); Wei et al. (2017); Wang, Kim, Guo, Zhao (2020).

8.2 RESPONSIVE AND PROGRAMMABLE MATERIALS FOR 4D PRINTING

4D-printing technology is in infancy and still continuously growing. A proper choice of materials as the printing inks is foremost essential in 4D printing. Researchers have explored different types of materials such as polymers, metals, ceramics, and their composites for 4D-printing applications. Although they have been extensively used in traditional 3D printing, due to the insufficient response to external stimuli most of the metals and ceramics are not suitable for 4D printing except limited ceramics (Liu, Zhao, Wu, & Lu, 2018) or shape-memory alloys (Caputo, Berkowitz, Armstrong, Müllner, & Solomon, 2018; Lu et al., 2019). The mainstream materials for the 4D printing are the responsive and programmable polymers and their composites due to the advantages of their easy processing, diverse stimulus responsiveness, large deformability, and widely tunable

material properties (molecular weight, crosslinking degree, amorphous/crystalline ratio, and molecular shape) (Delaey, Dubruel, & Van Vlierberghe, 2020; Pinho, Buga, & Piedade, 2020). Therefore this chapter focuses on 4D printing based on responsive and programmable polymers and their composites. Among a variety of them used for 4D printing, there are mainly two categories: shape-memory polymers (SMPs) and responsive shape-changing polymers and their composites (Momeni et al., 2017; Rastogi & Kandasubramanian, 2019; Wan, Luo, Liu, & Leng, 2020). These two types of smart materials used in 4D printing are summarized with explanations of their morphing mechanisms as follows.

8.2.1 SHAPE-MEMORY POLYMERS

SMPs are a group of polymers that can produce large recoverable deformation upon typically heat stimulation (Behl & Lendlein, 2011). SMPs were first mentioned in the 1940s (Vernon & Vernon, 1941). Since then, the main work in SMPs has been from the industry until the early 2000s when the research effort started due to their potential applications in biomedicine (Lendlein & Langer, 2002). Shape-memory effect of SMPs arises from the phase transformation of switching domains or segments which show glass–rubber and/or crystal–amorphous transitions (Liu, Qin, & Mather, 2007). The mechanism is illustrated in Figure 8.2. A temporary shape can be programmed by the material and alterations from a permanent shape. This temporary shape can be retained as long as the environmental temperature is below the transition temperature (T_{trans}) and then recover to its permanent shape upon the stimulation of heat (Behl & Lendlein, 2011; Mather, Luo, & Rousseau,

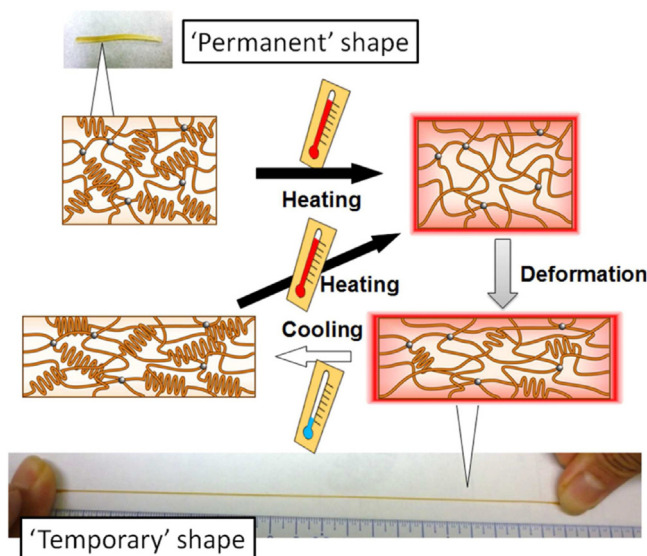


FIGURE 8.2

Molecular mechanism of the thermally induced shape-memory effect.

Adapted from Ebara (2015).

2009; Meng & Li, 2013). The shape-transformation process of a typical SMP normally contains the following steps. First, an SMP is deformed to a desired temporary shape by applying external mechanical force at a temperature above its T_{trans} ; then, the deformed SMP is cooled down and unloaded to fix the temporary shape; finally, the SMP is again heated above its T_{trans} to recover its original and permanent shape. To realize such a shape-transformation behavior, SMPs must consist of two segments, a highly elastic matrix and the phase changing domain capable of reducing its stiffness under certain external stimuli. Upon stimulation, the phase changing in SMPs can be triggered, which allows for the storage or release of the strain energy inside to program their temporary shapes or recovery to their permanent shapes.

Shape-memory behavior of polymer materials directly impacts the final performance of the 4D-printed SMP devices. It quantifies the degree of the applied strain that can be recovered by the shape-memory effect (Delaey et al., 2020). The strain recovery (R_r)—which indicates the capability of an SMP to retain its permanent shape—and shape fixity (R_f)—which indicates the degree that an SMP can retain its temporary shape in stress-free conditions—are the two widely used quantification indicators. The calculation of R_r is based on a “per cycle” compared with the previous cycle (as shown in Eq. (8.1)) or for N th cycles compared to the original permanent shape (as shown in Eq. (8.2)). The calculation of R_f is shown in Eq. (8.3).

$$R_r(N) = \frac{\varepsilon_m - \varepsilon_p(N)}{\varepsilon_m - \varepsilon_p(N-1)} \quad (8.1)$$

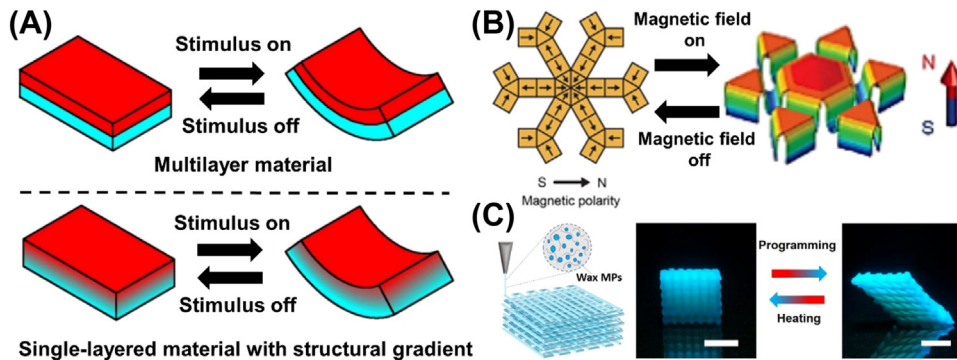
$$R_{r,\text{tot}}(N) = \frac{\varepsilon_m - \varepsilon_p(N)}{\varepsilon_m} \quad (8.2)$$

$$R_f(N) = \frac{\varepsilon_u(N) - \varepsilon_p(N-1)}{\varepsilon_m - \varepsilon_p(N-1)} \quad (8.3)$$

where ε_m refers to the maximum strain applied to the sample in % while $\varepsilon_p(N)$ is the residual strain in the permanent shape after N cycles; and $\varepsilon_u(N)$ refers to the strain retained in the stress-free state after unloading the sample in the N th cycle. Details on quantifying more shape-memory behaviors of SMPs can be found in this review article (Sauter, Heuchel, Kratz, & Lendlein, 2013).

8.2.2 RESPONSIVE SHAPE-CHANGING POLYMERS AND THEIR COMPOSITES

The responsive shape-changing polymers (RSCPs) are structurally reversible materials, which can spontaneously transform their shapes upon environmental stimuli and recover to their original shapes upon removal of the stimuli (Liu, Genzer, & Dickey, 2016; Van Manen, Janbaz, & Zadpoor, 2018). According to the different shape-transformation mechanisms, the RSCPs can be divided into the following subclasses. The shape transformation of the first type of RSCPs is driven by anisotropic volume changes inside the materials. Such anisotropy can be attributed to inhomogeneous swelling, thermal expansion, crystallization, or molecular reconfiguration upon external stimuli (Behl, Kratz, Zotzmann, Nöchel, & Lendlein, 2013; Deng, Dong, et al., 2018; Deng, Zhang, et al., 2018; Ube, Kawasaki, & Ikeda, 2016). The anisotropies in RSCPs can be created during or/and after the fabrication process by various approaches. One approach is to construct bilayer or multilayer structures with anisotropic properties in each layer (Kempaiah & Nie, 2014). The other approach is to create spatially controlled physical or chemical inhomogeneity in single-layered

**FIGURE 8.3**

Shape-transformation mechanism of the responsive shape-changing polymers, which are driven by (A) anisotropic volume changes, (B) magnetic force, and (C) phase change of elastomer composites.

(A) Adapted from [Van Manen et al. \(2018\)](#); (B) Adapted from [Zhao et al. \(2019\)](#); (C) Adapted from [Deng, Zhang, et al. \(2020\)](#).

RSCPs ([Deng et al., 2017](#); [Kempaiah & Nie, 2014](#)). Due to their anisotropic properties, distinct volume change will be generated in these smart materials when responding to the same stimuli. As a result, the mismatch in their volume changes generates internal stresses, offering driving forces for micro- to macroscaled shape changes. The shape changes of the second type of RSCPs are driven by magnetic force ([Figure 8.3B](#)). This type of RSCPs is often composed of magnetic particles embedded in a polymer matrix ([Deng, Sattari, et al., 2020](#); [Kim, Yuk, Zhao, Chester, & Zhao, 2018](#); [Xu et al., 2019](#)). The magnetic particles enable to create nonuniform magnetization profiles inside the composite materials. The nonuniform magnetization profiles generate magnetic forces and torques in different directions in response to an actuation magnetic field, driving the shape transformation of the magnetic composite materials ([Zhao, Kim, Chester, Sharma, & Zhao, 2019](#)). The shape transformation can also be resulting from the phase change of RSCP composites consisting of elastomers and phase change materials ([Deng, Xu, et al., 2020](#); [Deng, Zhang, et al., 2020](#)) ([Figure 8.3C](#)). Phase change leads to anisotropic residual strain in the prestretched RSCP composites. The applied strains can be quantitatively controlled to result in multistable shape-morphing structures. Another type of shape transformation is enabled by hydraulic or pneumatic inflation ([Shintake, Caccucciolo, Floreano, & Shea, 2018](#)). This mechanism often involves RSCPs that are often flexible and inflatable materials that can be used to fabricate deformable chambers. The shape transformation is obtained through the exerted pressure by a fluid (liquid or gas) on the chambers ([Sun, Song, & Paik, 2013](#)).

8.3 REALIZATION OF 4D PRINTING

The various research topics in 4D printing can fall into either development of new responsive and programmable material inks or the development of new 3D-printing techniques or both. Thus 4D printing is closely related to 3D printing whose advancement provides new possibilities for

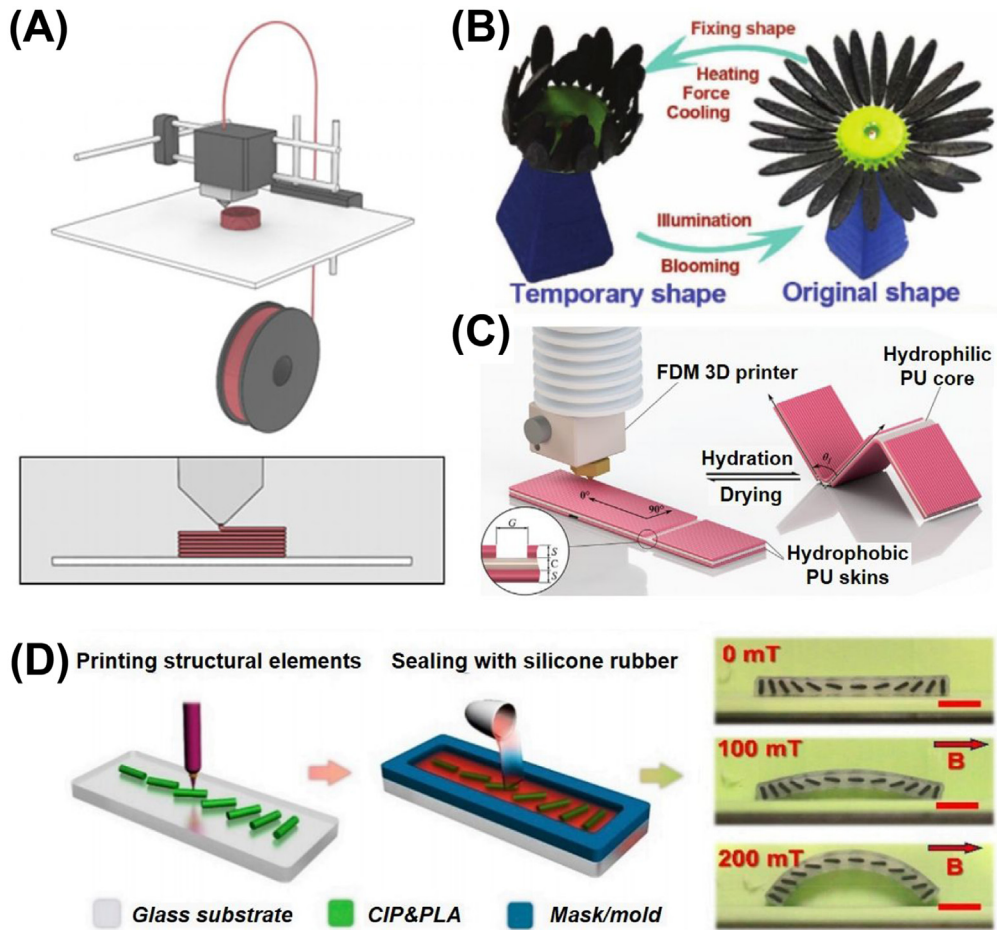
promoting 4D printing. At present, the 3D-printing techniques commonly adopted for 4D printing can be classified into three main categories, including fusion deposition modeling (FDM), direct ink writing (DIW), and vat photopolymerization (VP) (Shafranek et al., 2019). In this section, the technical characteristics of each printing technique will be introduced followed by a discussion of several implementation cases which adopt these printing techniques to print some representative responsive and programmable materials.

8.3.1 4D PRINTING BASED ON FUSION DEPOSITION MODELING

FDM, also called fused filament fabrication, is a commonly used 3D-printing technique. During the printing process, solid and thermoplastic filaments are melted and extruded from a heated nozzle (Figure 8.4A). Then the extruded materials follow a predetermined trajectory to form a 3D object in a line-by-line and a layer-by-layer manner. The printing materials should be able to flow out the printing head after heated and then be quickly solidified once printed. Based on this technical feature, FDM is widely used for 4D printing of SMPs, RSCPs as well as their composites.

Yang et al. demonstrated 3D printing of polyurethane (PU), a thermoplastic SMP, by FDM (Yang, Chen, Wei, & Li, 2016). The commercial PU pellets, DiAPLEX MM-4520 from SMP Technologies, Inc, were first processed to be printable filament by an extruder. Then the filament was fed to an FDM 3D printer to print 3D shapes including flowers and grippers. These printed 3D shapes showed a good shape-memory effect, in which the flower petals or gripper arms could fold together when heated above their T_{trans} . SMP composites can also be printed by the FDM method. Senatov et al. fabricated a shape-memory PLA/hydroxyapatite (HA) composite scaffold by FDM (Senatov et al., 2016). The additive HA efficiently enhanced the mechanical property of the composites to increase the fixity of the permanent shape, meanwhile realizing 98% shape recovery upon being heated. In addition to improving the mechanical property of the SMP composites, the additives empowered other functionalities. For instance, Yang et al. reported the FDM printing of a PU/carbon black composite to fabricate a photoresponsive device (Yang et al., 2017). Due to the photo-thermal effect of the carbon black, light acted as an environmental stimulus to trigger the shape recovery of the printed shapes. As shown in Figure 8.4B, the printed photoresponsive flower was programmed into a temporary shape and then blossomed to its printed permanent shape once exposed to the light.

FDM was also employed to print RSCPs. For example, Baker et al. demonstrated origami-inspired multilayer structures printed by FDM. They exhibited reversible shape-transformation behaviors when triggered by water (Figure 8.4C; Baker et al., 2019). The FDM can well print the active PU hydrogel layer and the passive PU elastomer layer. The shape change of this origami-inspired structure was achieved by the mismatched volume change of the two layers where the active layer exhibited much more volume expansion when absorbing water. Correa et al. reported 4D printing of self-morphing wood by FDM (David Correa et al., 2015). The “Wood” filaments used for FDM were developed by mixing wood microfibers with a printable polymer. The wood microfibers remained hygroscopically active, which swelled or shrank in response to humidity change. The induced shear stress during the printing aligned the wood microfibers along the longitudinal direction of the extrusion path of the nozzle. As a result, the printed responsive wood transformed its shapes when the relative humidity changed. Various shape transformations could be programmed by controlling the alignment of wood microfibers in the composites. In addition to

**FIGURE 8.4**

(A) Schematic of fused deposition modeling (FDM). (B) Photoresponsive shape-memory behavior of an FDM-printed blooming sunflower. (C) FDM-printed origami-inspired multilayer structures, which exhibited reversible shape-transformation behaviors triggered by water. (D) Fabrication and shape-transformation behavior of FDM-printed magneto-active soft materials.

(A) Adapted from [Shafranek et al. \(2019\)](#); (B) Adapted from [Yang et al. \(2017\)](#); (C) Adapted from [Baker et al. \(2019\)](#); (D) Adapted from [Qi et al. \(2020\)](#).

fabricating hygroscopic RSCPs, FDM can also print magnetic RSCPs. Qi et al. demonstrated 4D printing of a shape-programmable magneto-active soft material (MASM) ([Figure 8.4D](#)) ([Qi et al., 2020](#)). The magnetic carbonyl iron particles (CIPs) were mixed with polylactic acid (PLA) to form printable filaments for FDM. The shapes were printed from the CIPs/PLA filaments and then encapsulated within a silicone rubber. The printing could well control the magnetic regions with

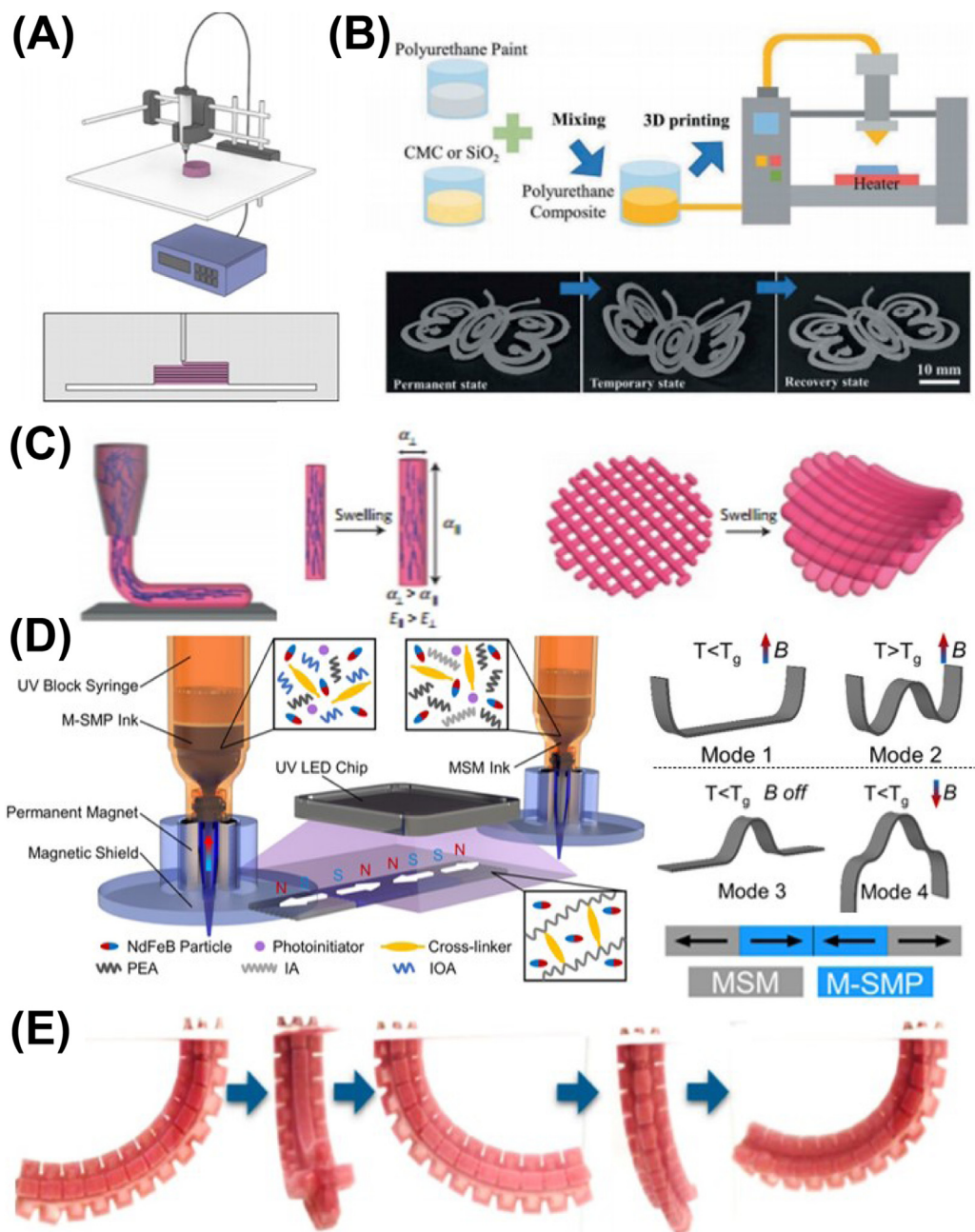


FIGURE 8.5

(Continued)

any shape, distribution, and orientation to generate anisotropic magnetization profiles to realize MASMs based microrobots with functions of walking, swimming, and snatching.

8.3.2 4D PRINTING BY DIRECT INK WRITING

DIW is one of the widely used 3D-printing processes based on the layer-by-layer extrusion principle to construct microstructures from a dispenser filled with printing ink (Figure 8.5A). During this process, a viscous ink that can be cured later is deposited line-by-line and layer-by-layer to form a 3D object. The conditions for the extrusion process include proper shear stress-induced thinning and suitable rheological properties. The DIW technique has the advantage of allowing customized materials, low materials consumption, open-source of controlling, and feasibility for multimaterials printing.

DIW has shown great potential for printing SMPs. For example, Su et al. reported DIW printing of polyurethane paint-based SM composites (Su et al., 2019). In this work, Su et al. present a simple and inexpensive additive method to print waterborne polyurethane (PU) paint mixed with either carboxymethyl cellulose (CMC) or silicon oxide (SiO_2) nanoparticles (NPs) (Figure 8.5B). These additives improved the rheological properties of the polyurethane paint for making printable ink, which improved the printing resolution and enhanced additive manufacturability. The printed PU SM objects exhibited time-resolved shape transformation upon heat stimulation. Wei et al. reported the DIW printing of remotely actuated SM objects from a UV cross-linkable poly(lactic acid) (PLA)/ Fe_3O_4 composite ink (Wei et al., 2017). By utilizing the hysteresis effect of Fe_3O_4 NPs under an alternating magnetic field, the PLA matrix was remotely heated to trigger the shape change. In contrast to thermoplastic SMPs, thermoset SMPs are usually quite difficult to be 3D printed. If they are cured before printing, they are hard to be extruded out of a printing nozzle. If cured after printing, the printed structures are usually weak and may easily collapse (Lei et al., 2019). To solve this problem, Zhang et al. recently demonstrated 4D printing of thermoset poly(glycerol dodecanoate) acrylate by a DIW technique (Zhang et al., 2020), an SMP with a shape-memory effect that can be triggered by human body temperature (Zhang, Deng, et al., 2019). To achieve structural integrity, the authors introduced photo-active groups in the prepolymer chains for photo-crosslinking prior to thermal condensation reaction.

DIW can also print RSCPs. Gladman et al. developed a biomimetic DIW-printing method to print a cellulose fibril contained hydrogel precursor ink into programmable bilayer architectures (Figure 8.5C) (Sydney Gladman, Matsumoto, Nuzzo, Mahadevan, & Lewis, 2016). During printing, the cellulose fibrils were aligned by the induced shear as the ink flowed through the printing nozzle, leading to spatially resolved anisotropic stiffness: the one in the longitudinal direction was larger than the one in the transverse direction. As a result, the anisotropic swelling behavior

- ◀ (A) Schematic of direct ink writing (DIW). (B) Fabrication and shape-memory behavior of DIW-printed polyurethane paint-based composites. (C) Fabrication and shape-transformation behavior of DIW-printed hydrogel driven by anisotropic swelling. (D) Fabrication and shape-transformation behavior of DIW-printed magneto-active soft materials. (E) Shape-transformation behavior of a DIW-printed hydraulic actuator.

(A) Adapted from Shafranek et al. (2019); (B) Adapted from Su et al. (2019); (C) Adapted from Sydney Gladman et al. (2016); (D) Adapted from Ma et al. (2020); (E) Adapted from Cheng et al. (2019).

controlled by the alignment of the cellulose fibrils along prescribed printing pathways was established. When the printed subjects uptook water, the swelling in the longitudinal direction was smaller than in the transverse direction. By harnessing such anisotropic swelling in the hydrogel composite, complicated and precisely controlled shape transformation in the printed structures was realized. The efficacy of such a printing method relies on the ability to deterministically defining the elastic and swelling anisotropies by local control of the orientation of the cellulose fibrils within the hydrogel.

Another type of model materials that DIW can employ for 4D printing are the magnetic RSCPs (Kim et al., 2018; Ma et al., 2020; Wu et al., 2020). A pioneer work was done by Kim et al. in 2018, which reported 4D printing of untethered fast-transforming MASMs by a magnetic field-assisted DIW (Kim et al., 2018). In a representative magnetic printer, a magnetic field was applied to the dispensing nozzle (Figure 8.5D). The inks used in these works were often polymer composites containing ferromagnetic NdFeB microparticles (MPs). During the printing process, the NdFeB MPs were reoriented along the applied magnetic field so that the magnetic polarity in the printed filaments was encoded. Such a manufacturing approach allowed to program ferromagnetic domains in the printed structures, leading to complex and fast shape transformations of the printed 3D shapes under the magnetic actuation. The remote actuation of such untethered, complex, and fast shape-shifting MASMs shows potential applications in flexible electronics, biomedical devices, and soft robotics (Kim et al., 2018).

Last but not the least, the DIW technology can also be used to fabricate hydraulic or pneumatic actuators. Cheng et al. developed a support-aided DIW method to fabricate a hydrogel hydraulic tentacle (Cheng et al., 2019). The hydraulic tentacle featured four channels composed of embedded chambers on four sides. To print such a hydraulic tentacle which showed a suspended structure, a functional polyacrylamide (PAAM) ink was printed on an alginate support part. After curing, the support part was removed by water, creating chambers inside the PAAM hydrogel. After water was injected into channels of the hydraulic tentacle, the chamber was inflated, resulting in the expansion of surrounding hydrogel walls. The asymmetric extension drove the unidirectional bending in the opposite direction of the inflated channel. A full circle rotation movement of the tentacle was achieved by programming the time-dependent output of injected water volume into the channels (Figure 8.4E). Truby et al. reported multimaterial DIW printing of the pneumatic actuators innervated with a complex network of sensors (Truby et al., 2018). Conductive ionogel and fugitive inks were printed within the three elastomeric matrices to define the sensor and pneumatic networks required for sensing and actuation, respectively. This manufacturing approach enabled the seamless integration of multiple ionically conductive and fluidic features within the elastomeric matrices to create the pneumatic actuators with the sensing capability.

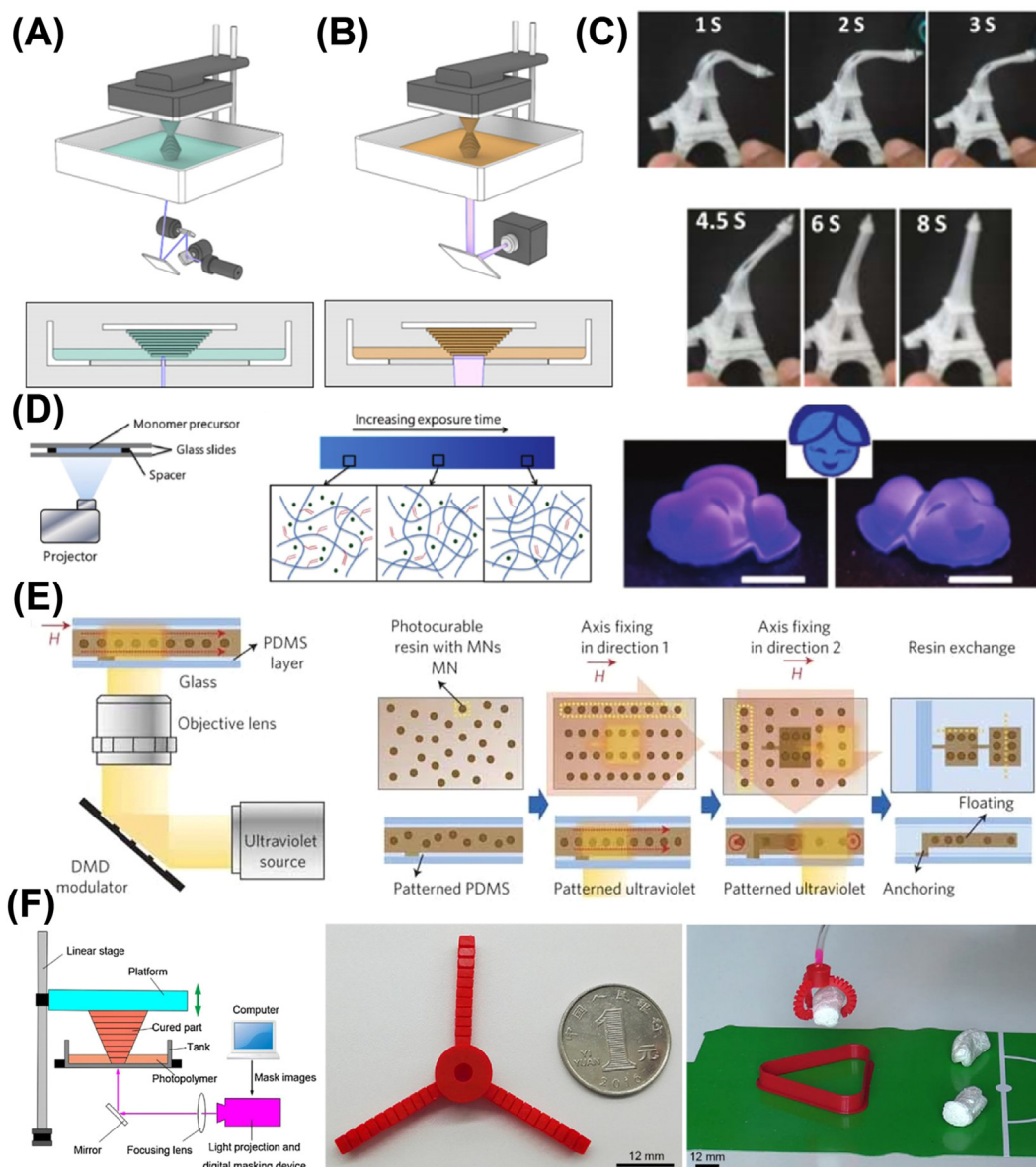
8.3.3 4D PRINTING BY PHOTOPOLYMERIZATION

Photopolymerization (VP) is currently the most widely used rapid 3D-printing method. It is characterized by high spatial resolution and high fabrication speed. Generally speaking, during the process of VP, a liquid resin is selectively polymerized and cross-linked into a solidified polymer induced by local laser sources. After the first layer is printed, a new layer is introduced and afterward cross-linked by laser illumination. The 3D object is finished by repetitively depositing resin layer by layer. VP can be mainly classified into stereolithography (SLA) and digital light processing (DLP).

The difference between them is that SLA uses a laser point to solidify part of the object's layer at a time (Figure 8.6A) whereas DLP solidifies an entire layer of the object using a digital mask (Figure 8.6B). There is a trade-off between these two approaches when considering the printing resolution and printing speed. DLP is superior to SLA in printing speed while SLA has higher resolution. In the past decade, both SLA and DLP have been used for 4D printing various responsive and programmable materials.

SMPs have been printed by the VP-based 3D-printing techniques. Yu et al. exploited SLA to print shape-memory objects from a photo-active acrylate-epoxy copolymer (Yu et al., 2017). The printed 3D structures exhibited good shape-memory behavior as proven by fold-deploy experiments and shape-memory cycling tests. The shape fixity and recovery ratio after 10 test cycles were still 99% and 100%, respectively. Figure 8.6C shows a shape recovery process of an Eiffel Tower model printed by SLA. The deformed model recovered to its original shape in 8 s when heated above its T_{trans} . Zarek et al. demonstrated printing of shape-memory polycaprolactone (PCL) objects by DLP (Zarek et al., 2016). In this work, methacrylate PCL oligomers were melted in a heating vat and then photo-cross-linked into high-resolution 3D shape-memory structures using a commercial DLP printer. To demonstrate the applicability of such a 4D-printing process, they printed several complex structures, which exhibited good shape-memory behaviors. A shape of a bird was fabricated and then programmed into a temporary shape with folded wings. The wings were unfolded after being heated above T_{trans} . In addition to printing single SMPs, SLA or DLP can also print shape-programmable structures from multiple SMPs. Ge et al. demonstrated the fabrication of shape-memory structures from photocurable methacrylate copolymers that formed polymer networks via free radical photopolymerization (Ge et al., 2016). The fabrication was enabled by a high-resolution projection microstereolithography system with an automated printing ink exchanger. As the properties of the different SMPs were different, a time-dependent sequential shape recovery was realized in the printed structures. The sequential recovery of a printed flower shape was demonstrated by first raising the temperature to 50°C to trigger shape deployment of the outer petals followed by a temperature rise to 70°C, which stimulated the subsequent opening of the inner petals.

SLA or DLP can be used to print RSCPs as well. Huang et al. developed a DLP method to 4D-printing shape-changing materials, which were responsive to water (Figure 8.6D) (Huang et al., 2017). A photocurable resin was composed of hydroxyethyl acrylate (HEA), hydroxyethyl methacrylate (HEMA), and potassium 3-sulfopropyl-methacrylate (PSPMA). A digital projector in the visible light wavelength range has been utilized for the curing process. They found that by modulating the exposure duration in space the crosslinking degrees and swelling properties of the hydrogel were spatially controlled. A longer exposure time resulted in a structure with a smaller swelling ratio. Due to the anisotropic swelling properties, the printed 2D films were evolved into complex 3D structures when immersed in water. Yuan et al. reported a versatile multimaterial DLP-based 4D-printing method that enabled the transformation of the printed 2D patterns to complex 3D structures (Yuan, Wang, & Ge, 2021). It was realized by printing an elastomeric layer and a covalently bonded hydrogel layer. The high water content of the hydrogel led to a large volumetric shrinkage and great modulus escalation after dehydration. The dehydration resulted in a volumetric shrinkage of up to 60% and an increased modulus from 100 kPa to 4 GPa. The printed patterns evolved into complex 3D structures with large bending curvatures and high bending stiffnesses.

**FIGURE 8.6**

Schematic of (A) stereolithography (SLA) and (B) digital light processing (DLP). (C) Shape-memory behavior of an SLA-printed Eiffel tower. (D) Fabrication and shape-transformation behavior of a DLP-printed hydrogel structure, which exhibited reversible shape-transformation behavior trigger by water. (E) Fabrication of a DLP-printed magneto-active soft material. (F) Fabrication and shape-transformation behavior of a DLP-printed pneumatic actuator.

(A, B) Adapted from *Shafranek et al. (2019)*; (C) Adapted from *Yu et al. (2017)*; (D) Adapted from *Huang et al. (2017)*; (E) Adapted from *Kim et al. (2011)*; (F) Adapted from *Ge et al. (2018)*.

The VP-based 3D printing can also fabricate objects from the magnetic RSCPs (Kim et al., 2011; Xu et al., 2019). For instance, Kim et al. developed a magnetic field-assisted DLP method to encode 3D magnetization in a magnetic responsive composite at the submillimeter scale (Figure 8.6E) (Kim et al., 2011). The resin used for the 3D printing was composed of a UV curable elastomer and magnetic NPs. The DLP system included a cube permanent magnet that was employed to generate a magnetic field to precisely reorient the magnetic NPs encapsulated in the matrix of the UV curable elastomer. After the magnetic NPs reorientation, a DLP projector emitted UV light on selected regions of the resin to initiate polymerization and froze the magnetic particles within the selected regions, thus realigning the chains of magnetic NPs and inducing cooperative torque. Such a two-step fabrication procedure was repeated to generate the desirable magnetic anisotropy in the printed objects. In this way, the printed magnetic responsive structures exhibited predesigned, complex two- and three-dimensional motions. Last but not the least, VP-based 3D printing has been shown to fabricate pneumatic actuators. For instance, Ge et al. built a DLP 3D printer that showed high printing speed and printed objects with a feature size as small as 87.5 μm (Ge, Dong, Wang, Ge, & Gu, 2018) (Figure 8.6F). The microfluidic pneumatic grippers with dimensions of 0.4 mm $\times$ 0.4 mm could be printed within 30 minutes. They could generate sufficient large deformation for grasping objects under 20 kPa input air pressure.

8.4 APPLICATIONS OF 4D PRINTING

8.4.1 BIOMEDICAL APPLICATIONS

8.4.1.1 Tissue Engineering

4D printing has emerged as a useful tool for tissue engineering. The advantages of 4D printing used in tissue engineering include (1) construction of 3D complex tissue constructs based on responsive materials that are able to reshape or change their functions according to environmental stimuli and (2) postmaturation of printed cell population in the scaffolds with programmable architectures, enabling generation of the tissue constructs with functionalities that are similar to those of native tissues (Gao et al., 2016; Shie et al., 2019).

Dui et al. 4D printed a cardiac patch with a smart design of physiological adaptability from photocurable hydrogels by using an SLA printing technique (Cui et al., 2020). The 4D printing was able to achieve many essential requirements in manufacturing tissue micropatterns and architectures. The crosslinking degree in the hydrogel was controlled by the printing speed and laser intensity. As a result, the induced internal stress gradient arising from the anisotropic crosslinking degree drove the shape morphing of the printed patch. The programmed self-morphing made the patch conform to the surface of the heart. Moreover, the fiber arrangement in the highly stretchable hydrogel patch enabled to switch from a wavy pattern to a mesh pattern in accordance with the switch between diastole and systole in the cardiac cycle. Such design efficiently increased the tolerance of the printed hydrogel cardiac patch when exposed to dynamic mechanics for providing a physiologically tunable matrix environment for implantation.

Miao et al. reported a 4D-printed SMP substrate for studying differentiation behaviors of stem cells (Miao et al., 2020). The substrate was made of soybean oil epoxidized acrylate. The fabrication process involved printing and imprinting of micropatterns. The SMP showed excellent cell

compatibility and significantly improved cardiomyogenic differentiation of the stem cells. By triggering the morphing of the micropatterns in the SMPs substrate, the differentiation behaviors were effectively regulated, which showed great potential for osteochondral and neural tissue engineering.

8.4.1.2 Implantable Devices

4D printing has shown a new application for fabricating SMP-based stents for minimizing the surgical invasion of the implantation through their shape-memory effect (Yeazel & Becker, 2020). The stents are firstly fabricated as the desired geometries that fit the dimension of the target and then are programmed to a smaller size for ease of implantation. After the implantation, the stents recover to their original dimension upon external triggers. The 4D printing allows for quick fabrication of customized stents with patient-specific geometries. For instance, Ge et al. reported 4D printing of an SMP stent using high-resolution DLP (Ge et al., 2016). Wei et al. demonstrated DIW of the SMP stents by UV curable poly(lactic acid) (PLA)/Fe₃O₄ composite (Wei et al., 2017). The magneto-responsive SMPs stents were remotely guided to the implantation destination by a magnetic field, and then deployed by the heat generated from the hysteresis effect of Fe₃O₄ nanoparticles under another applied oscillated magnetic field (Figure 8.7). Besides 4D printing of the stents for blood vessel devices, 4D printing would show great potential for developing the occlusion devices for treating cardiac diseases (Huang, Kong, & Venkatraman, 2014).

4D printing has also demonstrated an emerging application for fabricating bone tissue constructs in which the shape- and function-transformation can meet the need of personalized bone regeneration by dynamically adapting to the usually irregular defect areas (Wan, Zhang, Liu, Lv, & Zhou, 2020). In addition, the spatiotemporal response of the shape transformation would make the distributions and release of bioactive cues possible for establishing biomimetic microenvironments, which promote regeneration of complex heterogeneous bone, vascular, and nerve tissues in the defective areas. Toward this goal, Miao et al. reported 3D SLA-based bioprinting of photocurable soybean oil epoxidized acrylate resins for biocompatible scaffolds that supported the growth of

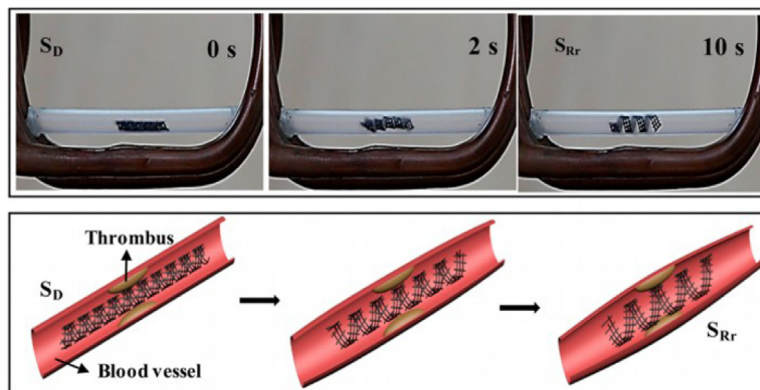


FIGURE 8.7

A 4D-printed scaffold as an intravascular stent.

Adapted from Wei et al. (2017).

multipotent human bone marrow mesenchymal stem cells (Miao et al., 2016). The printed scaffolds could be programmed and then recovered to original shapes at human body temperature (37°C) for adapting the geometries of the bone defective areas, indicating potentials for 4D printing. Xuan et al. in their recent work developed physiological-temperature-triggered shape-memory ternary scaffolds from a copolymer of poly(glycerol sebacate) (PGS) and poly(1,3-propylene sebacate) (PPS) for cell-free cartilage repair (Xuan et al., 2020). The novelty of the work was that the materials maintained good elasticity and shape-memory response meanwhile showing chondrogenic capacity endowed by the immobilized bioactive kartogenin (KGN) in the scaffolds. They showed great potential for minimally invasive implantation.

8.4.2 SOFT ROBOTS

4D printing can be used to fabricate soft pneumatic robots. For instance, Zhang et al. printed miniature soft pneumatic robots through SLA technology (Zhang, Ng, et al., 2019). They explored the soft robots in the application as a soft debris remover, which navigated a confined space and then collected small objects in a hard-to-reach position. Such functions opened the application field that traditional robots made of rigid materials may not be suitable. For instance, due to lack of flexibility collecting small objects that are ingested into engines by the rigid robots can cause damages to the engine structure while a soft robot which was composed of a continuum manipulator and a 3D-printed soft pneumatic gripper can circumvent such a potential problem. The continuum manipulator could navigate the space while the pneumatic gripper could grasp and lift the object. As shown in Figure 8.8, a mockup was constructed to simulate the situation of removing the debris trapped in the engines. The soft robot navigated through two holes controlled by the continuum manipulator and then grasped and removed the object without causing collateral damage to the structure.

In addition to the pneumatic soft robot fabrication, 4D printing was also exploited for fabricating magnetic soft robots (Kim et al., 2018; Xu et al., 2019). Kim et al. demonstrated a hexapedal magnetic soft robot which could stop and hold a fast-moving object under a magnetic field

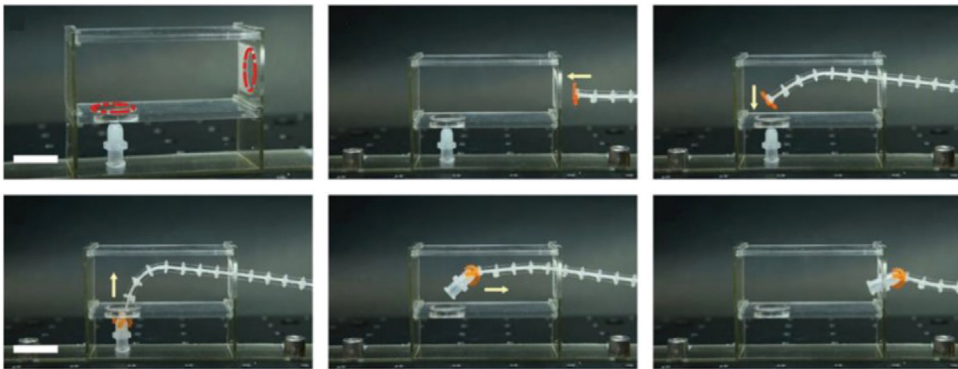


FIGURE 8.8

A 4D-printed miniature soft pneumatic robot showing a function as a soft debris remover.

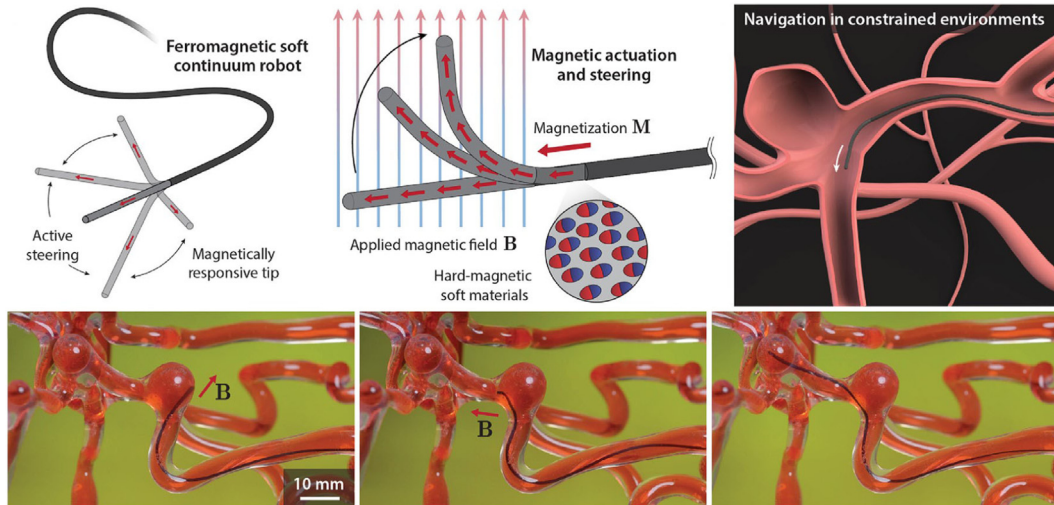
Adapted from Zhang, Ng, et al. (2019)

generated by a permanent magnet. The hexapedal magnetic soft robot could also carry a pharmaceutical pill by using a rolling locomotion upon application of a rotating magnetic field (Kim et al., 2018). Xu et al. demonstrated an untethered and magnetic millimeter-scale soft robot by a magnetic field-assisted DIP technology (Xu et al., 2019). An eight-legged paddle-crawling robot was fabricated by integrating magnetic NdFeB MPs into the matrix of UV curable elastomer. During the printing process, alternating magnetization was encoded in the legs of the robot. As a result, the robot could stand on the legs and move forward using the stroke action under a rotating magnetic field. The stroke action involved the arms moving forward alternately with the legs kicking the ground throughout the stroke. The paddle-crawling motion of the multilegged robot was presented under a rotating magnetic field. Moreover, by controlling the magnetic field through a joystick controller, the controlled motion of crawling and turning in a confined space was realized.

Due to the advantage of remote actuation in enclosed and confined spaces, 4D-printed magnetic soft robots show potential in biomedical applications for minimally invasive surgery. For instance, Kim et al. reported a DIW-printed soft continuum robot with omnidirectional steering and navigating capabilities based on magnetic actuation (Kim, Parada, Liu, & Zhao, 2019; Wang et al., 2020). The robot was composed of a soft polymer body composited with uniformly dispersed ferromagnetic microparticles and a hydrogel skin grown on its surface. During the printing process, ferromagnetic domains were programmed in the soft body of the robot to be used for omnidirectional navigation under an external magnetic field. The hydrogel skin could effectively decrease the surface friction. With all these features, the soft robot could navigate complex and constrained spaces, such as a cerebrovascular phantom with multiple aneurysms, through active, omnidirectional steering motions driven by magnetic actuation (Figure 8.9). These motions are usually difficult to be achieved by the bulky and rigid robotic catheters or passive manual instruments. These soft continuum robots open new avenues to minimally invasive robotic surgery for previously inaccessible lesions, thereby addressing challenges and unmet needs in healthcare.

8.4.3 FLEXIBLE ELECTRONICS

4D printing was also explored for application in flexible electronics. Rodriguez et al. printed SMPs based on multifunctional flexible electronics by DIW (Rodriguez et al., 2016). The flexible electronics was printed from SMP ink containing conductive carbon nanofibers (CNFs). The temporary shape of the 3D-printed SMP object resulted in an open electrical circuit. When the SMP was heated above T_{trans} , the shape was recovered to close the circuit, thus lighting up an LED (Figure 8.10A). More interestingly, the shape change could be also actuated by applying electric stimulus to the SMP/CNFs composite. As shown in the thermal images, the resistive heating from a power source of 20 V, 150 mA induces the shape change from the programmed shape to the printed shape in 180 seconds. Wan et al. reported the 4D printing of electro-responsive shape-changing sensors to detect various liquids (Wan, Zhang, Liu, & Leng, 2019). The sensors were printed from inks made of poly(D,L-lactide-co-trimethylene carbonate) (PLMC)/CNT composites by DIW. The working mechanism of the devices is illustrated as follows (Figure 8.10B). When the level of a target liquid decreased, the contact area of a sensor that was immersed in liquid was decreased, thus lowering the detection precision. To maintain the detection precision, adaptive shape-changing of the sensor to keep the contact area between the sensor and liquid was realized by electrically stimulating the printed PLMC/CNT. Under 25 V within 16 seconds, the flat sensor was transformed to a

**FIGURE 8.9**

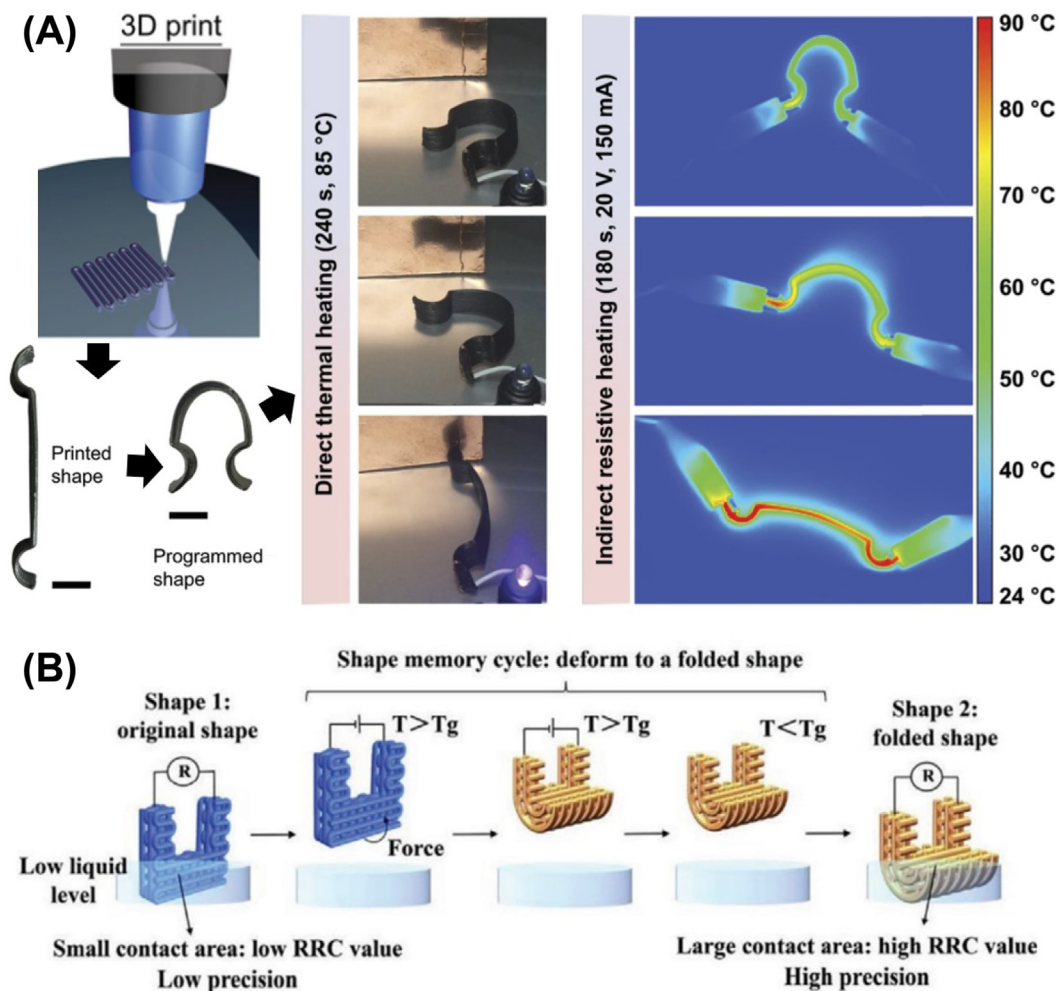
A 4D-printed magnetic soft robotic for minimally invasive surgery.

Adapted from Wang et al. (2020).

folded one to increase its contact with the liquid when the liquid level was decreased, thus maintaining a high relative resistance change. It is envisioned that 4D printing would open a new avenue to fabricating shape-changing sensors with environmental adaptability for detecting leakage of various solvents in varying environments.

8.4.4 FOOD PROCESSING

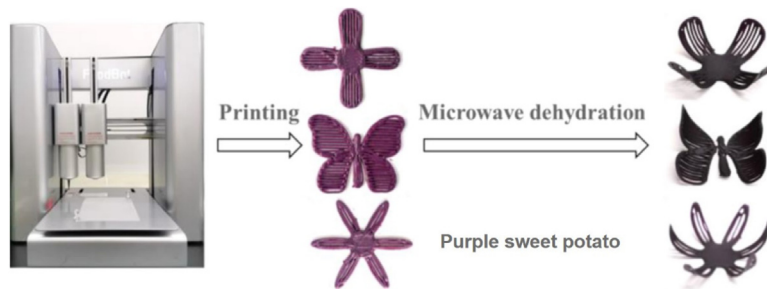
Finally, 4D printing was also exploited in applications for food processing (He, Zhang, Devahastin, 2020; He, Zhang, & Fang, 2020). A pioneer work was done by Wang et al. (2017). They developed a type of transformative food by DIW-printing approach. The printable food ink was composed of common food ingredients such as protein, cellulose, and starch. Through 3D printing, the printable food ink was processed into edible planar films with anisotropic swelling proprieties. By absorbing water, these planar films were transformed into complex 3D structures. Other than swelling, dehydration could also induce the programmable shape transformation of 3D printed food. For instance, He et al. adopted microwave heating to dehydrate the bilayered 3D printed purple sweet potato purees (He, Zhang, Devahastin, 2020). The water loss in the printed sample would result in a moisture gradient inside the 3D printed food, which in turn caused the internal stress and drive the spontaneous deformation (Figure 8.11). The shape changing of these foods brings in desired sensorial characteristics to improve appetite by demonstrating the futuristic dining experience via interaction design. In addition, 4D food printing has the advantage of substantially reducing shipping costs and storage space by first the “flat packaging” and then on-demand 3D shape deployment.

**FIGURE 8.10**

(A) The application of a 4D-printed shape-memory device for flexible electronics. (B) Schematic illustration of a shape-changing sensor with high detection precision when the liquid level decreased.

(A) Adapted from *Rodriguez et al. (2016)*; (B) Adapted from *Wan et al. (2019)*.

In addition to bringing the shape-changing concept into the 4D food printing, evolution of properties, for example, color appearance and flavor, of 3D-printed food over time (fourth dimension) has recently attracted much attention (*Chen, Zhang, Guo, & Chen, 2021; Ghazal, Zhang, & Liu, 2019; He, Zhang, Guo, 2020; Phuhongsung, Zhang, & Bhandari, 2020*). They 3D-printed anthocyanin-potato starch and anthocyanin gel. Anthocyanin is a smart material whose color change can be triggered by PH stimulus (*Ghazal et al., 2019*). In the work, the lemon solution with different

**FIGURE 8.11**

The application of 4D printing for fabricating transformative foods.

Adapted from He, Zhang, & Fang, 2020.

concentrations acted as the PH stimulus. Later a different food ingredient of mashed potato/purple sweet potato puree was explored for 4D printing of color-evolved food (He, Zhang, Guo, 2020). In addition to the PH stimulus, microwave which induces heat in the food was applied to stimulate the color change of 3D-printed curcumin lotus root gel (Chen et al., 2021). The deprotonation of curcumin due to the heat generated by the microwave resulted in the color change from yellow to red. Moreover, the same research group also demonstrated that the microwave as an external thermal stimulus could induce the automatic flavor change of postprinted food made from soybean protein isolate (SPI), k-carrageenan (CAR), and vanilla flavor (VNL) (Phuhongsung et al., 2020).

8.5 CONCLUSION AND PROSPECTIVE

4D printing has emerged as a technology of fabricating structures and devices displaying a wide range of length scales and functionalities. It has shown a wide range of potential applications in extensive fields including biomedicine, robotics, electronics, and food processing. However, challenges remain owing to inherent problems related to various aspects including printable materials, printing throughput and resolution, multimaterial printing, and simulation tools for structural design and processing optimization. In order to make the 4D-printing technology more ready for the transition from the lab to market, these aspects are worth being further investigated.

First, there is a continuous need of developing novel smart materials for 4D printing. New chemistries for crosslinking and new explorations into photochemical responses during or after printing will lead to smart materials with enhanced properties for 4D printing. The development of new smart materials is contributed to address fundamental 4D-printing issues such as avoiding needs of supporting structures for printing suspending structures, lowering the cost of the printable materials, and increasing the printing speed. Beyond the pure synthetic materials, the emerging field of applying living organisms for material design and synthesis opens new opportunities for 4D printing (Lu, Aimetti, Langer, & Gu, 2017). The synthetic biology that aims to program biological systems to perform user-defined functions provides a new platform for editing living materials, which can be explored for 4D printing (Tang et al., 2020).

Second, improving the current 3D-printing technologies or developing new printing techniques are highly demanded to advance 4D printing. The advancement includes higher printing resolution, higher printing speed, and enabled multimaterials fabrication. For instance, two-photon lithography can realize a sub-100 nm resolution (Meza, Das, & Greer, 2014; Meza et al., 2015). It was applied to print responsive 3D structures with the feature size in the sub-10 μm . In addition, a much narrower selection of materials can be effectively operated in current 3D-printing techniques, thereby making printed 3D structures have almost no access to materials (e.g., semiconductors and metals) and their resulting devices. Thus the development of a hybridized 3D printer with multimaterials printing capability would be a possible technological route to achieving multifunctionalities and realizing multiple shape morphing modes in the printed systems (Peng et al., 2021).

Third, new mathematic tools are needed for designing and predicting the shape-transformation behaviors of the 4D-printed structures. To rationally design and predict the shape-transformation process of the 4D-printed structures, finite element analysis simulation has been widely employed. However, the shape-transformation process in 4D-printed structures is dynamic and time-dependent, often featuring far-from-equilibrium dynamics and involving nonlinearity and complexity, which could exhibit too many degrees of freedom to be fully decoded by the physics-based modeling. Recently emerged data-driven machine learning methods may offer an alternative solution for this challenging problem, which has been presented in several pioneer works (Hamel et al., 2019; Su et al., 2020) and is worth for further investigation. Other systematic computational frameworks for the design of structures will also open new opportunities in 4D printing (Dimassi et al., 2021; Gu et al., 2020; Yang et al., 2020).

QUESTIONS

1. What is 4D printing? Please use an example to illustrate the difference of 3D printing and 4D printing.
2. What is shape-changing mechanism for SMPs?
3. Please give at least three main groups of responsive and programmable materials and in each group with two specific materials to illustrate their shape or property change mechanism.
4. Please illustrate the advantages and disadvantages of FDM-, DIW-, and VP-based 3D-printing techniques when applied to 4D printing.
5. What are the basic requirements when 4D printing is applied to fabricate implantable biomedical devices?
6. Please describe a process enabled by 4D printing for fabricating a magnetic-active soft robot.

References

- Baker, A. B., Bates, S. R. G., Llewellyn-Jones, T. M., Valori, L. P. B., Dicker, M. P. M., & Trask, R. S. (2019). 4D printing with robust thermoplastic polyurethane hydrogel-elastomer trilayers. *Materials & Design*, 163, 107544.
- Behl, M., Kratz, K., Zotzmann, J., Nöchel, U., & Lendlein, A. (2013). Reversible bidirectional shape-memory polymers. *Advanced Materials*, 25, 4466–4469.

- Behl, M., & Lendlein, A. (2011). Shape-memory polymers. *Kirk-Othmer Encyclopedia of Chemical Technology*, 1–16.
- Caputo, M. P., Berkowitz, A. E., Armstrong, A., Müllner, P., & Solomon, C. V. (2018). 4D printing of net shape parts made from Ni-Mn-Ga magnetic shape-memory alloys. *Additive Manufacturing*, 21, 579–588.
- Chen, C., Zhang, M., Guo, C., & Chen, H. (2021). 4D printing of lotus root powder gel: Color change induced by microwave. *Innovative Food Science and Emerging Technologies*, 68, 102605.
- Cheng, Y., Chan, K. H., Wang, X.-Q., Ding, T., Li, T., Lu, X., & Ho, G. W. (2019). Direct-ink-write 3D printing of hydrogels into biomimetic soft robots. *ACS Nano*, 13, 13176–13184.
- Chu, H., Yang, W., Sun, L., Cai, S., Yang, R., Liang, W., ... Liu, L. (2020). 4D printing: A review on recent progresses. *Micromachines*, 11, 796.
- Cui, H., Liu, C., Esworthy, T., Huang, Y., Yu, Z.-X., Zhou, X., ... Zhang, L. G. (2020). 4D physiologically adaptable cardiac patch: A 4-month *in vivo* study for the treatment of myocardial infarction. *Science Advances*, 6, eabb5067.
- David Correa, A. P., Guberan, C., Jhaveri, N., Reichert, S., Menges, A., & Tibbits, S. (2015). 3D-printed wood: Programming hygroscopic material transformations. *3D Printing and Additive Manufacturing*, 2, 106–116.
- Delaey, J., Dubruel, P., & Van Vlierberghe, S. (2020). Shape-memory polymers for biomedical applications. *Advanced Functional Materials*, 30. Available from <https://doi.org/10.1002/adfm.201909047>.
- Deng, H., Dong, Y., Su, J. W., Zhang, C., Xie, Y., Zhang, C., ... Lin, J. (2017). Bioinspired programmable polymer gel controlled by swellable guest medium. *ACS Applied Materials & Interfaces*, 9, 30900–30908.
- Deng, H., Dong, Y., Zhang, C., Xie, Y., Zhang, C., & Lin, J. (2018). An instant responsive polymer driven by anisotropy of crystal phases. *Materials Horizons*, 5, 99–107.
- Deng, H., Sattari, K., Xie, Y., Liao, P., Yan, Z., & Lin, J. (2020). Laser reprogramming magnetic anisotropy in soft composites for reconfigurable 3D shaping. *Nature Communications*, 11, 6325.
- Deng, H., Xu, X., Zhang, C., Su, J. W., Huang, G., & Lin, J. (2020). Reprogrammable 3D shaping from phase change microstructures in elastic composites. *ACS Applied Materials & Interfaces*, 12, 4014–4021.
- Deng, H., Zhang, C., Sattari, K., Ling, Y., Su, J. W., Yan, Z., & Lin, J. (2020). 4D printing elastic composites for strain-tailored multistable shape morphing. *ACS Applied Materials & Interfaces*. Available from <https://doi.org/10.1021/acsami.1020c17618>.
- Deng, H., Zhang, C., Su, J.-W., Xie, Y., Zhang, C., & Lin, J. (2018). Bioinspired multi-responsive soft actuators controlled by laser tailored graphene structures. *Journal of Materials Chemistry B*, 6, 5415–5423.
- Dimassi, S., Demoly, F., Cruz, C., Qi, H. J., Kim, K. Y., André, J. C., & Gomes, S. (2021). An ontology-based framework to formalize and represent 4D printing knowledge in design. *Computers in Industry*, 126, 103374.
- Ebara, M. (2015). Shape-memory surfaces for cell mechanobiology. *Science and Technology of Advanced Materials*, 16.
- Gao, B., Yang, Q., Zhao, X., Jin, G., Ma, Y., & Xu, F. (2016). 4D bioprinting for biomedical applications. *Trends in Biotechnology*, 34, 746–756.
- Ge, L., Dong, L., Wang, D., Ge, Q., & Gu, G. (2018). A digital light processing 3D printer for fast and high-precision fabrication of soft pneumatic actuators. *Sensors and Actuators A: Physical*, 273, 285–292.
- Ge, Q., Qi, H. J., & Dunn, M. L. (2013). Active materials by four-dimension printing. *Applied Physics Letters*, 103, 131901.
- Ge, Q., Sakhaei, A. H., Lee, H., Dunn, C. K., Fang, N. X., & Dunn, M. L. (2016). Multimaterial 4D printing with tailorable shape memory polymers. *Scientific Reports*, 6, 31110.
- Ghazal, A. M. F., Zhang, M., & Liu, Z. B. (2019). Spontaneous color change of 3D printed healthy food product over time after printing as a novel application for 4D food printing. *Food and Bioprocess Technology*, 12, 1627–1645.

- Gu, J., Narayanan, V., Wang, G., Luo, D., Jain, H., Lu, K., . . . Yao, L. (2020). Inverse design tool for asymmetrical self-rising surfaces with color texture. *Symposium on computational fabrication*, 1–12.
- Hamel, C. M., Roach, D. J., Long, K. N., Demoly, F., Dunn, M. L., & Qi, H. J. (2019). Machine-learning based design of active composite structures for 4D printing. *Smart Materials and Structures*, 28, 065005.
- He, C., Zhang, M., & Devahastin, S. (2020). Investigation on spontaneous shape change of 4D printed starch-based purees from purple sweet potatoes as induced by microwave dehydration. *ACS Applied Materials & Interfaces*, 12(34), 37896–37905.
- He, C., Zhang, M., & Fang, Z. (2020). 3D printing of food: Pretreatment and post-treatment of materials. *Critical Reviews in Food Science and Nutrition*, 60, 2379–2392.
- He, C., Zhang, M., & Guo, C. F. (2020). 4D printing of mashed potato/purple sweet potato puree with spontaneous color change. *Innovative Food Science and Emerging Technologies*, 59, 102250.
- Huang, L., Jiang, R., Wu, J., Song, J., Bai, H., Li, B., . . . Xie, T. (2017). Ultrafast digital printing toward 4D shape changing materials. *Advanced Materials*, 29, 1605390.
- Huang, Y. Y., Kong, J. F., & Venkatraman, S. S. (2014). Biomaterials and design in occlusion devices for cardiac defects: A review. *Acta Biomaterialia*, 10, 1088–1101.
- Kempaiah, R., & Nie, Z. (2014). From nature to synthetic systems: Shape transformation in soft materials. *Journal of Materials Chemistry B*, 2, 2357–2368.
- Kim, J., Chung, S. E., Choi, S. E., Lee, H., Kim, J., & Kwon, S. (2011). Programming magnetic anisotropy in polymeric microactuators. *Nature Materials*, 10(10), 747–752.
- Kim, Y., Parada, G. A., Liu, S., & Zhao, X. (2019). Ferromagnetic soft continuum robots. *Science Robotics*, 4, eaax7329.
- Kim, Y., Yuk, H., Zhao, R., Chester, S. A., & Zhao, X. (2018). Printing ferromagnetic domains for untethered fast-transforming soft materials. *Nature*, 558, 274–279.
- Kuang, X., Roach, D. J., Wu, J., Hamel, C. M., Ding, Z., Wang, T., . . . Qi, H. J. (2019). Advances in 4D printing: Materials and applications. *Advanced Functional Materials*, 29, 1805290.
- Lei, D., Yang, Y., Liu, Z., Chen, S., Song, B., Shen, A., . . . You, Z. (2019). A general strategy of 3D printing thermosets for diverse applications. *Materials Horizons*, 6, 394–404.
- Leist, S. K., & Zhou, J. (2016). Current status of 4D printing technology and the potential of light-reactive smart materials as 4D printable materials. *Virtual and Physical Prototyping*, 11, 249–262.
- Lendlein, A., & Langer, R. (2002). Biodegradable, elastic shape-memory polymers for potential biomedical applications. *Science (New York, N.Y.)*, 296, 1673–1676.
- Liu, C., Qin, H., & Mather, P. T. (2007). Review of progress in shape-memory polymers. *Journal of Materials Chemistry*, 17, 1543–1558.
- Liu, G., Zhao, Y., Wu, G., & Lu, J. (2018). Origami and 4D printing of elastomer-derived ceramic structures. *Science Advances*, 4, eaat0641.
- Liu, Y., Genzer, J., & Dickey, M. D. (2016). “2D or not 2D”: Shape-programming polymer sheets. *Progress in Polymer Science*, 52, 79–106.
- Lu, H. Z., Yang, C., Luo, X., Ma, H. W., Song, B., Li, Y. Y., & Zhang, L. C. (2019). Ultrahigh-performance TiNi shape memory alloy by 4D printing. *Materials Science and Engineering: A*, 763, 138166.
- Lu, Y., Aimetti, A. A., Langer, R., & Gu, Z. (2017). Bioresponsive materials. *Nature Reviews Materials*, 2, 16075.
- Ma, C., Wu, S., Ze, Q., Kuang, X., Zhang, R., Qi, H. J., & Zhao, R. (2020). Magnetic multimaterial printing for multimodal shape transformation with tunable properties and shiftable mechanical behaviors. *ACS Applied Materials & Interfaces*, 13(11), 12639–12648.
- Mather, P. T., Luo, X., & Rousseau, I. A. (2009). Shape memory polymer research. *Annual Review of Materials Research*, 39, 445–471.

- Meng, H., & Li, G. (2013). A review of stimuli-responsive shape memory polymer composites. *Polymer*, *54*, 2199–2221.
- Meza, L. R., Das, S., & Greer, J. R. (2014). Strong, lightweight, and recoverable three-dimensional ceramic nanolattices. *Science (New York, N.Y.)*, *345*, 1322–1326.
- Meza, L. R., Zelhofer, A. J., Clarke, N., Mateos, A. J., Kochmann, D. M., & Greer, J. R. (2015). Resilient 3D hierarchical architected metamaterials. *Proceedings of the National Academy of Sciences of the United States of America*, *112*, 11502–11507.
- Miao, S., Cui, H., Esworthy, T., Mahadik, B., Lee, S.-J., Zhou, X., ... Zhang, L. G. (2020). 4D self-morphing culture substrate for modulating cell differentiation. *Advanced Science*, *7*, 1902403.
- Miao, S., Zhu, W., Castro, N. J., Nowicki, M., Zhou, X., Cui, H., ... Zhang, L. G. (2016). 4D printing smart biomedical scaffolds with novel soybean oil epoxidized acrylate. *Scientific reports*, *6*, 27226.
- Momeni, F., Mehdi, S. M., Hassani, N., Liu, X., & Ni, J. (2017). A review of 4D printing. *Materials & Design*, *122*, 42–79.
- Peng, X., Kuang, X., Roach, D. J., Wang, Y., Hamel, C. M., Lu, C., & Qi, H. J. (2021). Integrating digital light processing with direct ink writing for hybrid 3D printing of functional structures and devices. *Additive Manufacturing*, *40*, 101911.
- Phuhongsung, P., Zhang, M., & Bhandari, B. (2020). 4D printing of products based on soy protein isolate via microwave heating for flavor development. *Food Research International*, *137*, 109605.
- Pinho, A. C., Buga, C. S., & Piedade, A. P. (2020). The chemistry behind 4D printing. *Applied Materials Today*, *19*, 100611.
- Qi, S., Guo, H., Fu, J., Xie, Y., Zhu, M., & Yu, M. (2020). 3D printed shape-programmable magneto-active soft matter for biomimetic applications. *Composites Science and Technology*, *188*, 107973.
- Quanjin, M., Rejab, M. R. M., Idris, M. S., Kumar, N. M., Abdullah, M. H., & Reddy, G. R. (2020). Recent 3D and 4D intelligent printing technologies: A comparative review and future perspective. *Procedia Computer Science*, *167*, 1210–1219.
- Rastogi, P., & Kandasubramanian, B. (2019). Breakthrough in the printing tactics for stimuli-responsive materials: 4D printing. *Chemical Engineering Journal*, *366*, 264–304.
- Rodriguez, J. N., Zhu, C., Duoss, E. B., Wilson, T. S., Spadaccini, C. M., & Lewicki, J. P. (2016). Shape-morphing composites with designed micro-architectures. *Scientific Reports*, *6*(1), 1–10.
- Sauter, T., Heuchel, M., Kratz, K., & Lendlein, A. (2013). Quantifying the shape-memory effect of polymers by cyclic thermomechanical tests. *Polymer Reviews*, *53*, 6–40.
- Senatov, F. S., Niaza, K. V., Zadorozhnyy, M. Y., Maksimkin, A. V., Kaloshkin, S. D., & Estrin, Y. Z. (2016). Mechanical properties and shape memory effect of 3D-printed PLA-based porous scaffolds. *Journal of the Mechanical Behavior of Biomedical Materials*, *57*, 139–148.
- Shafranek, R. T., Millik, S. C., Smith, P. T., Lee, C.-U., Boydston, A. J., & Nelson, A. (2019). Stimuli-responsive materials in additive manufacturing. *Progress in Polymer Science*, *93*, 36–67.
- Shie, M.-Y., Shen, Y.-F., Astuti, S. D., Lee, A. K.-X., Lin, S.-H., Dwijaksana, N. L. B., & Chen, Y.-W. (2019). Review of polymeric materials in 4D printing biomedical applications. *Polymers*, *11*, 1864.
- Shintake, J., Cacucciolo, V., Floreano, D., & Shea, H. (2018). Soft robotic grippers. *Advanced Materials*, *30*, 1707035.
- Su, J.-W., Gao, W., Trinh, K., Kenderes, S. M., Tekin Pulatsu, E., Zhang, C., ... Lin, J. (2019). 4D printing of polyurethane paint-based composites. *International Journal of Smart and Nano Materials*, *10*, 237–248.
- Su, J.-W., Li, D., Xie, Y., Zhou, T., Gao, W., Deng, H., ... Lin, J. (2020). A machine learning workflow for 4D printing: Understand and predict morphing behaviors of printed active structures. *Smart Materials and Structures*, *30*, 015028.
- Sun, Y., Song, Y. S., & Paik, J. (2013). Characterization of silicone rubber based soft pneumatic actuators. *2013 IEEE/RSJ international conference on intelligent robots and systems*, 4446–4453.

- Sydney Gladman, A., Matsumoto, E. A., Nuzzo, R. G., Mahadevan, L., & Lewis, J. A. (2016). Biomimetic 4D printing. *Nature Materials*, 15, 413–418.
- Tang, T. C., An, B. L., Huang, Y. Y., Vasikaran, S., Wang, Y. Y., Jiang, X. Y., ... Zhong, C. (2020). Materials design by synthetic biology. *Nature Reviews Materials*. Available from <https://doi.org/10.1038/s41578-020-00265-w>.
- Tibbitts, S. (2012). Design to self-assembly. *Architectural Design*, 82, 68–73.
- Tibbitts, S. (2014). 4D printing: Multi-material shape change. *Architectural Design*, 84, 116–121.
- Tibbitts, S., & Cheung, K. (2012). Programmable materials for architectural assembly and automation. *Assembly Autom*, 32, 216–225.
- Truby, R. L., Wehner, M., Grosskopf, A. K., Vogt, D. M., Uzel, S. G. M., Wood, R. J., & Lewis, J. A. (2018). Soft somatosensitive actuators via embedded 3D printing. *Advanced Materials*, 30, 1706383.
- Ube, T., Kawasaki, K., & Ikeda, T. (2016). Photomobile liquid-crystalline elastomers with rearrangeable networks. *Advanced Materials*, 28, 8212–8217.
- Van Manen, T., Janbaz, S., & Zadpoor, A. A. (2018). Programming the shape-shifting of flat soft matter. *Materials Today*, 21, 144–163.
- Vernon, L. B., Vernon, H. M. (1941). Process of manufacturing articles of thermoplastic synthetic resins. *United States Patent No. 2,234,993*.
- Wan, X., Luo, L., Liu, Y., & Leng, J. (2020). Direct ink writing based 4D printing of materials and their applications. *Advanced Science*, 7, 2001000.
- Wan, X., Zhang, F., Liu, Y., & Leng, J. (2019). CNT-based electro-responsive shape memory functionalized 3D printed nanocomposites for liquid sensors. *Carbon*, 155, 77–87.
- Wan, Z. Q., Zhang, P., Liu, Y. S., Lv, L. W., & Zhou, Y. S. (2020). Four-dimensional bioprinting: Current developments and applications in bone tissue engineering. *Acta Biomaterialia*, 101, 26–42.
- Wang, L., Kim, Y., Guo, C. F., & Zhao, X. (2020). Hard-magnetic elastica. *Journal of the Mechanics and Physics of Solids*, 142, 104045.
- Wang, W., Yao, L., Zhang, T., Cheng, C.-Y., Levine, D., & Ishii, H. (2017). *Transformative appetite: Shape-changing food transforms from 2D to 3D by water interaction through cooking. Proceedings of the 2017 CHI conference on human factors in computing systems* (pp. 6123–6132). Association for Computing Machinery.
- Wei, H., Zhang, Q., Yao, Y., Liu, L., Liu, Y., & Leng, J. (2017). Direct-write fabrication of 4D active shape-changing structures based on a shape memory polymer and its nanocomposite. *ACS Applied Materials & Interfaces*, 9, 876–883.
- Wu, S., Hamel, C. M., Ze, Q., Yang, F., Qi, H. J., & Zhao, R. (2020). Evolutionary algorithm—guided voxel—encoding printing of functional hard—magnetic soft active materials. *Advanced Intelligent Systems*, 2 (8), 2000060.
- Xu, T., Zhang, J., Salehizadeh, M., Onaizah, O., & Diller, E. (2019). Millimeter-scale flexible robots with programmable three-dimensional magnetization and motions. *Science Robotics*, 4, eaav4494.
- Xuan, H., Hu, H., Geng, C., Song, J., Shen, Y., Lei, D., ... You, Z. (2020). Biofunctionalized chondrogenic shape-memory ternary scaffolds for efficient cell-free cartilage regeneration. *Acta Biomaterialia*, 105, 97–110.
- Yang, H., Leow, W. R., Wang, T., Wang, J., Yu, J., He, K., ... Chen, X. (2017). 3D printed photoresponsive devices based on shape memory composites. *Advanced Materials*, 29, 1701627.
- Yang, H., Qian, K., Liu, H., Yu, Y., Gu, J., McGehee, M., ... Yao, L. (2020). Simulearn: Fast and accurate simulator to support morphing materials design and workflows. *Proceedings of the 33rd annual ACM symposium on user interface software and technology*, 71–84.
- Yang, Y., Chen, Y., Wei, Y., & Li, Y. (2016). 3D printing of shape memory polymer for functional part fabrication. *The International Journal of Advanced Manufacturing Technology*, 84, 2079–2095.

- Yeazel, T. R., & Becker, M. L. (2020). Advancing toward 3D printing of bioresorbable shape memory polymer stents. *Biomacromolecules*, 21, 3957–3965.
- Yu, R., Yang, X., Zhang, Y., Zhao, X., Wu, X., Zhao, T., . . . Huang, W. (2017). Three-dimensional printing of shape memory composites with epoxy-acrylate hybrid photopolymer. *ACS Applied Materials & Interfaces*, 9, 1820–1829.
- Yuan, C., Wang, F., & Ge, Q. (2021). Multimaterial direct 4D printing of high stiffness structures with large bending curvature. *Extreme Mechanics Letters*, 42, 101122.
- Zarek, M., Layani, M., Cooperstein, I., Sachyani, E., Cohn, D., & Magdassi, S. (2016). 3D printing of shape memory polymers for flexible electronic devices. *Advanced Materials*, 28, 4449–4454.
- Zhang, C., Cai, D., Liao, P., Su, J. W., Deng, H., Vardhanabhuti, B., . . . Lin, J. (2020). 4D Printing of shape-memory polymeric scaffolds for adaptive biomedical implantation. *Acta Biomaterialia*, 122, 101–110.
- Zhang, C., Deng, H., Kenderes, S., Su, J. W., Whittington, A., & Lin, J. (2019). Chemically interconnected thermotropic polymers for transparency-tunable and impact-resistant windows. *ACS Applied Materials & Interfaces*, 11, 5393–5400.
- Zhang, Y.-F., Ng, C. J.-X., Chen, Z., Zhang, W., Panjwani, S., Kowsari, K., . . . Ge, Q. (2019). Miniature pneumatic actuators for soft robots by high-resolution multimaterial 3D printing. *Advanced Materials Technologies*, 4, 1900427.
- Zhao, R., Kim, Y., Chester, S. A., Sharma, P., & Zhao, X. (2019). Mechanics of hard-magnetic soft materials. *Journal of the Mechanics and Physics of Solids*, 124, 244–263.
- Zhou, Y., Huang, W. M., Kang, S. F., Wu, X. L., Lu, H. B., Fu, J., & Cui, H. (2015). From 3D to 4D printing: Approaches and typical applications. *Journal of Mechanical Science and Technology*, 29, 4281–4288.

Blood Vessel Regeneration

9

Jesse K. Placone^{1,2}, Megan Kimicata^{3,4} and John P. Fisher^{2,4}

¹*Department of Physics and Engineering, West Chester University, West Chester, PA, United States*

²*Fischell Department of Bioengineering, University of Maryland, College Park, MD, United States*

³*Department of Materials Science and Engineering, University of Maryland, College Park, MD, United States*

⁴*Center for Engineering Complex Tissues, University of Maryland, College Park, MD, United States*

9.1 INTRODUCTION

In the field of tissue engineering, one of the most rapidly advancing areas of study is blood vessel regeneration. This area focuses on the development of technologies and methods to encourage either new vessel growth, angiogenesis, or the repair and replacement of native blood vessels. New vessel formation has specific applications to the survival of implanted organs as discussed in another chapter in this book. The replacement of native blood vessels has direct therapeutic applications due to the prevalence of cardiovascular disease worldwide and in the United States (Benjamin et al., 2017; Go et al., 2012; Roger et al., 2012). Cardiovascular disease and congenital heart disease have had significant advances in their treatment and care; however, the usage of synthetic materials still leaves much to be desired due to complications associated with their use. These artificial grafts typically have problems with the progressive occlusion of the implant because of a lack of growth potential and an increase in thromboembolic events (Cleveland et al., 1992; Jonas, Freed, Mayer, & Castaneda, 1985; Lamers et al., 2012; Stark, 1998). As such, the field of tissue engineering has been investigating multiple routes toward the development of a vascular graft that has the potential to overcome some of the shortcomings of traditional vascular grafts and improve the quality of life for the patient.

In this chapter, we will discuss some of the recent technologies that have been developed to address problems in this field, and each approach's potential future direction to become clinically relevant for cardiovascular disease treatments and for the implantation of other tissue-engineered implants. The current approaches can be divided into cell-based and cell-free systems where man-made scaffolds or grafts are generated to provide the foundation for blood vessel regeneration. Both approaches make use of micro- to nanoscale architecture, embedded or attached growth factors or therapeutics, and tunable mechanical properties to better mimic the native tissue and its heterogeneous composition of cells and extracellular matrices.

The most basic description of blood vessels typically illustrates the blood vessel as consisting of three main layers. These layers, the tunica intima, the tunica media, and the tunica adventitia, are organized as concentric layers from the inside out and all have distinct cellular and matrix

components. The innermost layer, the tunica intima, consists of an endothelial cell (EC) layer that completely covers inside of the blood vessel. These cells are critical for the control of hemostasis and maintaining the patency of the blood vessel. Additionally, these cells provide a layer that is selectively permeable while at the same time providing a lining that regulates platelet and leukocyte adhesion and aggregation, and influences smooth muscle cell migration and proliferation. The ECs in this region deposit and maintain a basement membrane of type IV collagen and laminin. There is an elastic laminin layer separating this layer from the next cell population. The middle layer, or tunica media, is characterized by a second cell type, smooth muscle cells (SMCs). These cells are organized concentrically and control vasoconstriction and vasodilatation. The outermost layer, the tunica adventitia, comprises primarily fibroblasts. This layer's extracellular matrix (ECM) is different from the other layers in that it is made up of collagen I, elastin, laminin, and fibronectin. The exact composition of this layer and the presence of various growth factors and cytokines greatly influence the mechanical strength and local cell response, and it varies based upon location and function in the body. These three layers work in tandem to control and modulate the physical and biological properties of the blood vessel. The recapitulation of these three main layers is critical to the success of a tissue-engineered vascular implant, and as such, much research has been carried out to develop manufacturing methods to address the heterogeneous nature of human vasculature.

9.1.1 ADDITIVE MANUFACTURING

To manufacture mimetic scaffolds for vascular applications, three-dimensional (3D) printing is increasingly being employed as it provides the ability to tailor scaffolds with stringent control of the material on a layer-by-layer basis. Additive manufacturing has been present in industrial applications, but has only recently become an emerging technology for use in tissue engineering applications (Barron, Wu, Ladouceur, & Ringeisen, 2004; Campbell & Weiss, 2007; Derby, 2012; Horn & Harrysson, 2012; Marga et al., 2012; Melchels et al., 2012; Schubert, van Langeveld, & Donoso, 2013; Xu, Jin, Gregory, Hickman, & Boland, 2005). However, other technologies exist that can mimic the native structures and architecture with a more global approach and as such will be briefly discussed in this chapter.

One critical aspect of the advancement of 3D printing, or rapid prototyping and additive manufacturing, with regard to tissue engineering is the development of vascularized tissues. Efficient vascularization would allow for incorporation into the host tissue as well as provide an avenue for necessary nutrient and waste exchange. The circulatory system is how the body naturally maintains homeostasis, transports waste and nutrients, delivers chemical signals, and provides rapid delivery of the hosts' defenses. It is, therefore, especially important that any implanted tissue-engineered construct incorporates vascularization in its design.

Advances in computer-aided design (CAD) have also had an impact on the rational design of tissue-engineered implants for vascular regeneration (Almeida Hde & Bartolo, 2010; Barron et al., 2004; Guillotin et al., 2010; Hirt, Hansen, & Eschenhagen, 2014; Horn & Harrysson, 2012; Jakab et al., 2010). As mentioned previously, the main cellular component of the inner layer of vasculature is ECs. These ECs maintain homeostasis through the formation of a boundary reinforced with their cell–cell junctions and their deposited ECM, and secrete various growth factors to control proliferation and cell recruitment, and induce ECM production. In this regard, CAD approaches

have been beneficial because they can be used to model the shear stress that the ECs will be exposed to upon implantation into the body. It is well known that EC protein expression is modulated by shear stress, and by controlling the microenvironment that the cells are exposed to, the expression of proteins such as platelet-derived growth factor (PDGF), transforming growth factor- β (TGF β), and endothelial nitric oxide synthase (eNOS) can be manipulated. These proteins and cytokines can be utilized to help with the endothelialization process as well as maintain the implant integrity after implantation.

9.1.2 IMPORTANT PROTEINS FOR VASCULATURE

PDGF is known to play a role in the regulation of cell growth and proliferation. Specifically, PDGF is important for angiogenesis and has been the focus of many tissue engineering applications that wish to make use of existing vasculature to invade into a tissue-engineered implant. PDGF can act as chemoattractant for fibroblasts and influence the division of vascular SMCs.

TGF β is involved in the production of ECM by modulating the production of elastin (Sales et al., 2006). Elastin itself has two main functions in the ECM. First, it is critical for the elastic properties of the blood vessel (Fernández-Colino et al., 2019; Kozel, Mecham, & Rosenbloom, 2011). The development of tissue-engineered materials or implants that mimic the elastic properties is very difficult but it is absolutely necessary to prevent complications such as neointimal hyperplasia, which is generally caused by an elastic mismatch between the native tissue and the implanted vascular graft (Creager, Loscalzo, & Beckman, 2012). The second function that elastin performs is the sequestration and activation of secreted growth factors (Creager et al., 2012). Therefore the presence of elastin at varying concentrations natively controls the activity of the proteins present in the ECM and modulates their release.

eNOS is critical for the functioning of an intact endothelial layer in blood vessels. It is involved in the generation of nitric oxide (NO) by ECs, which is important in the regulation of vascular resistance. NO produced by ECs can cause the relaxation of the SMCs surrounding the vasculature thereby resulting in vasodilatation. However, if the endothelium is disrupted or damaged, the expression of eNOS is altered and vasoconstriction can occur due to the decreased levels of NO. Additionally, the expression of eNOS can affect vessel permeability through the levels of NO produced (Dauphinee & Karsan, 2010).

Although there are many proteins and cytokines known to influence angiogenesis and vasculogenesis, the deposition of these proteins alone cannot guide the invasion of vasculature or generate new vasculature. However, these proteins and others can be used in conjunction with materials and scaffolds designed for vascular regeneration. Traditionally, techniques such as electrospinning, molding, and vapor deposition have been used to create the materials that these growth factors and signaling molecules will be attached to for vascular applications (Roy, 2010). There has, however, been a push in the past several years to have more control over the surface topologies, the spatial organization of these surface features and modifications, as well as the direct seeding of cells on the vascular implant. As such, the goal of many tissue engineers is to develop a vascular implant that has tightly controlled features and mechanical properties, while at the same time has very heterogeneous properties to mimic the native vasculature.

9.1.3 APPLICATION TO VASCULAR IMPLANTS

To accomplish this goal, many researchers have been investigating the application of additive manufacturing to create replacement vasculature. In the circulatory system, there are many different components, which could be either replaced or mimicked using 3D printing. The three main classifications that we will discuss here are: the arteries, which provide flow to the tissue; veins, which provide a return flow from the tissue; and capillaries, which provide for the efficient exchange of waste and nutrients in the tissue. Arteries and veins have larger inner diameters, which range from 2 to 5 mm and have wall thickness on the order of 1 mm for arteries and 0.5 mm for veins. Additionally, there are structural differences between arteries and veins even though they have the same three basic layers. Blood vessels can be thought of as a concentric structure with each concentric ring comprised of different cells and matrices ([Figure 9.1](#)). ECs form the smallest-diameter layer (tunica intima), followed by SMCs (tunica media), and then fibroblasts and primarily collagen farther out forming the outermost layer (tunica adventitia) ([Iaizzo, 2005; Junqueira & Carneiro, 1995](#)).

The cells are embedded inside of extracellular matrices that are primarily comprised of proteins, glycoproteins, glycosaminoglycans, and proteoglycans. In between these layers, there can be a layer of elastin. However, the thickness of each layer and the composition of those layers can vary for arteries and veins. Arteries have more SMCs surrounding the ECs, whereas the veins have a decreased presence of smooth muscles cells resulting in the disparity in their wall thicknesses, as well as providing different mechanical properties and functionality. It is especially important for arteries to have a thicker layer of SMCs and elastin layers due to the active role arteries play in the circulatory system. Veins on the other hand have a diminished need for SMCs due to their role in the vascular system not requiring strong contractions, and as a result, the tunica media is not the thickest layer. However, veins do have valves periodically along their length that prevent the back

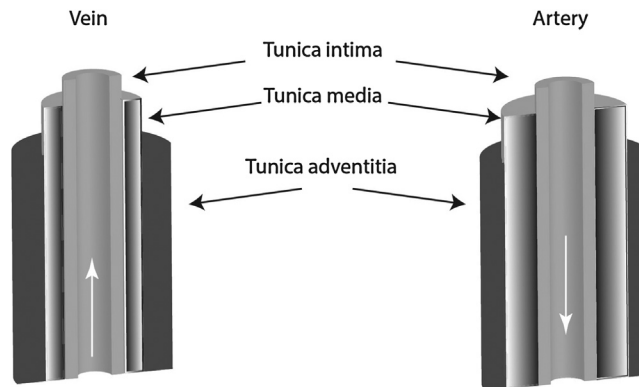


FIGURE 9.1

A cross-sectional view showing the basic layers of veins and arteries. The three main layers are labeled with black arrows. The tunica media and the tunica adventitia are different thicknesses for veins and arteries based upon their respective mechanical requirements. In between the tunica media and the inner and outer layers, there are thin layers of elastin matrix. The thickness of the elastin matrices is thicker in arteries.

flow of blood. These mechanical properties and functionality need to be taken into account when designing an implant for regenerative medicine (Iaizzo, 2005; Junqueira & Carneiro, 1995).

The vascular system is a very dynamic environment. Typically, the average adult human heart pumps 5 L of blood per minute throughout the body. Different organs and tissues receive varied quantities of flow depending on their nutrient requirements. These levels can also be dynamically modulated to account for various activities such as digestion, physical exertion, and basal metabolic activities. This provides a unique set of environmental conditions that are continually changing and as a result any implant must be able to survive this range of conditions.

To address the clinical need for biocompatible and biodegradable vascular grafts, tissue engineering approaches have become more widely utilized to improve vascular regeneration. These approaches are varied in the methods and are used to generate the grafts or scaffolds to promote vascularization and angiogenesis, but the main concepts behind the design of the grafts remain largely the same. The common features can be broken down into the components of the implant: (1) the scaffold, (2) functionalization, and (3) cells necessary for vascular regeneration. Some of the methods used by the tissue engineering community to fabricate these therapeutic implants are electrospinning, molding, and rapid prototyping of cell-free scaffolds and cell-based printed scaffolds. A selection of these methods, currently employed in scientific literature, will be discussed in the following sections with an emphasis on 3D printing technologies.

In the manufacturing of these implants, there are currently two main approaches, consisting either of a cell-free or a cell-based system of designing and creating the implants for vascular regeneration. These two approaches can be broken down further into the underlying technologies that allow for the creation of these tissue-engineered implants. The cell-free scaffolds are typically created using technologies such as electrospinning, chemical vapor deposition, and 3D printing. These methods are discussed in more depth in Section 9.2 of this chapter. Cell-based implants for vascular regeneration are produced either through the seeding of the cell-free scaffolds before implantation or through the direct creation of scaffolds where the cells are embedded in the scaffold during the production. The production of these cell-laden constructs is discussed in depth in Section 9.3 of this chapter and focuses primarily on current and emerging technologies for 3D printing cells for vascular regeneration applications.

9.2 CELL-FREE SCAFFOLDS

Investigations into blood vessel regeneration and growth have focused on the use of cell-free systems. In these tissue engineering endeavors, many technologies have been utilized to develop scaffolds that encourage the growth of native vasculature either *in vitro* or *in vivo*. Generally, these technologies aim to generate scaffolds that mimic the native vasculature and can be functionally modified as discussed previously to develop more bioactive scaffolds. This section aims to give an in-depth discussion of how the technologies work to generate the scaffolds and a few specific examples of how these methods are used in literature. Due to the number of methods available, this section discusses the following for generating scaffolds: (1) electrospinning, (2) stereolithography, and (3) fused-deposition modeling (FDM).

9.2.1 ELECTROSPINNING

This book focuses primarily on 3D printing and its application to various tissue engineering endeavors; however, the discussion of blood vessel regeneration would not be complete without a brief discussion of electrospinning. Nanofibrous electrospun grafts have been widely utilized for vascular regeneration applications. The electrospinning process allows for the control of graft parameters in real-time during the fabrication process, which is precisely why electrospun scaffolds have been investigated for multiple tissue engineering applications that are not limited to the vascular system (Fridrikh, Yu, Brenner, & Rutledge, 2003). There are a wide number of polymers and materials suitable for this fabrication process and the costs associated with this technology are relatively low. As a result of these two considerations, multiple studies have been carried out on a wide array of electrospun vascular grafts/scaffolds for tissue regeneration and the systems used to generate these scaffolds have been well characterized (Fu et al., 2014; Gao et al., 2016; Hadjizadeh, Ajji, Jolicoeur, Liberelle, & De Crescenzo, 2013; Han et al., 2013; Hashi et al., 2010; Lai et al., 2014; Rayatpisheh et al., 2014; Shapira, Kim, & Dvir, 2014; Wu, Fan, Chu, & Wu, 2010). Some unique advancements in electrospun vascular grafts include multilayer (Abdal-hay, Bartnikowski, Hamlet, & Ivanovski, 2018; Dong et al., 2018; Huang et al., 2018) and multimaterial (Ju et al., 2017; Montini-Ballarín et al., 2016; Tan, Wang, Gao, Liu, & Tan, 2016) processes, ECM incorporation (Gong et al., 2016; Wu et al., 2019), and drug treatment (Li et al., 2018; Norouzi & Shamloo, 2019; Qiu et al., 2017). However, the electrospinning system is not compatible with live cells due to the solvents typically used.

Typically, an electrospinning setup comprises a few main components: (1) a syringe filled with the desired polymer, (2) a syringe pump, (3) a high voltage power supply, and (4) a grounded collection plate/collector. See Figure 9.2 for an illustration. The syringe pump allows for the control

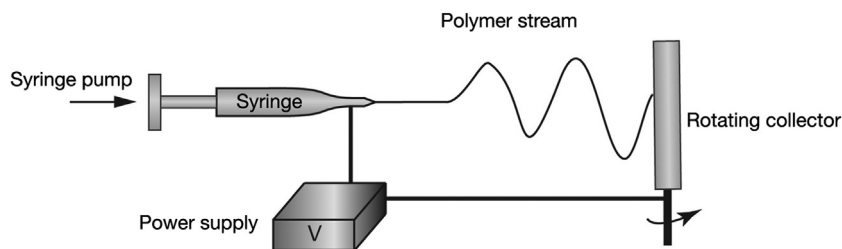


FIGURE 9.2

A typical electrospinning apparatus has four main components. (1) A high voltage power supply provides the potential difference required for the electrospinning process. The applied voltage can be modulated to vary the physical dimensions of the resulting fibers during the deposition process. (2) The syringe filled with the polymer solution and (3) the syringe pump provide the materials for the electrospinning process and control the stream produced via the flow rate and the needle gauge/type. (4) The collection plate or rotating cylindrical collector is where the deposited nanofiber mesh is deposited. By depositing directly onto a cylinder, the implants' size can be directly controlled and potentially used directly for blood vessel regeneration. If a flat collection plate is used, then the resulting electrospun fiber can be rolled into a cylinder of appropriate dimensions for use in the desired application.

of the flow rate, which can impact the size of the electrospun fibers. The syringe itself can have different types/gauges of needles to modulate the stream of the polymer being ejected from the syringe. Also, the voltage applied can be modulated to control the fiber diameter and provides the driving force due to electrostatic repulsion to encourage the polymer solution to flow toward the collection plate. There are additional external variables to consider, such as the temperature and humidity in the electrospinning chamber.

By modifying the applied voltage, the rotation velocity, and the solvent/polymer mixture, properties of the graft such as orientation, fiber diameter (hundreds of nanometers to microns), and density can be controlled. This allows for the control of properties such as porosity, mechanical strength, fiber alignment, and surface area (Deitzel, Kleinmeyer, Harris, & Beck Tan, 2001; Fridrikh et al., 2003).

In the case of polycaprolactone, groups have shown that using either a high rotation (2000 rpm) or low rotation (20 rpm) speed resulted in either aligned fibers or randomly deposited nanofibers, respectively. Furthermore, to better mimic the native vasculature and to recapitulate the layer variation as a function of radial distance, groups have shown that by simultaneously controlling the applied voltage and rotation speed, they are able to generate vascular graft material that comprises distinct layers with varying fiber orientation and alignment (Wu et al., 2010). This sort of electrospun tissue-engineered blood vessel has the potential to impact future designs of electrospun grafts for vascular applications because each layer's orientation can be controlled. Thus this allows the graft to physically mimic the orientation of the tunica intima and the tunica media which in turn can modulate the response of endothelial and vascular SMCs. By mimicking the morphology of the native tissue, these scaffolds show great promise for impacting vascular regeneration in tissue engineering; however, recapitulation of the morphology is only one aspect critical to successful vascular regeneration.

Current studies are investigating the modification of the scaffolds by incorporation of growth factors (Han et al., 2013; He, Yong, Teo, Ma, & Ramakrishna, 2005; He et al., 2012; Lai et al., 2014; Luong-Van et al., 2006; Montero et al., 2012; Ye et al., 2012; Zhang et al., 2013). It is important to note some of the general methods of modification of these grafts, specifically the direct incorporation of growth factors and the addition of secondary carrier molecules/materials to increase the regenerative properties of these acellular scaffolds. These molecules can be added directly to the electrospinning solution or can be physically bound to the implant surface through surface modification. As mentioned previously, the growth factors or proteins selected need to be carefully chosen for the desired application and positioned in the correct locations to elicit the desired cellular response in each layer of the implant. Given the low cost, multiple materials available, ease of fabrication, and the ability to tailor the surface of these electrospun implants (Soliman et al., 2011; Subbiah, Bhat, Tock, Parameswaran, & Ramkumar, 2005; Ziabari, Mottaghtalab, & Haghi, 2010), electrospinning will be a viable method of generating scaffolds for blood vessel regeneration for the foreseeable future.

9.2.2 STEREOLITHOGRAPHY

Another technique for the creation of cell-free scaffolds for vascular regeneration that has seen advances in the past several years is stereolithography. There are a few main technologies such as digital micromirror devices used in parallel with digital light processing that can be used with multiple light sources. One important aspect of this printing process is the fact that objects are rendered

layer-by-layer and as a result, the process is potentially faster than other 3D printing approaches. Furthermore, in terms of reducing the cost of the 3D printer and the overall cost to manufacture parts, stereolithography-based systems have a distinct advantage from other free-form printing methods because an XY motion controller is no longer required. As a result, only an accurate Z motion control system is needed which could reduce the system cost.

There are two typical methods used for stereolithography and for the generation of solid scaffolds for tissue engineering. The basic concept is the same but the source material reservoir and build plate behavior are different. In Figure 9.3, there are illustrations of these two printer setups. Each layer of the print can be thought of as horizontal slices through the final object where these slices are translated into images that are projected onto the printing resin. In the first printer setup, the layer's image is projected up through the bottom of the reservoir onto the build plate. After a layer is cured, a stepping motor moves the stage up the appropriate distance and the next layer is cured. There are several advantages to this system. First, the polymer solution flows back into the void left by the scaffold moving up with the stage; thus, there is no need for a pump to provide additional polymer solution, just what is in the resin reservoir is enough. Second, the photoinitiation of the polymerization process takes place in the solution and as a result is protected from oxygen, which may potentially adversely interact with the polymerization process. In the second printer setup, the projected image comes from the top, down onto the polymer solution/build plate. As each layer is cured, the build plate moves down and more printing resin is pumped in before the initiation of the next layer. One advantage of this system is that much larger prints can be produced

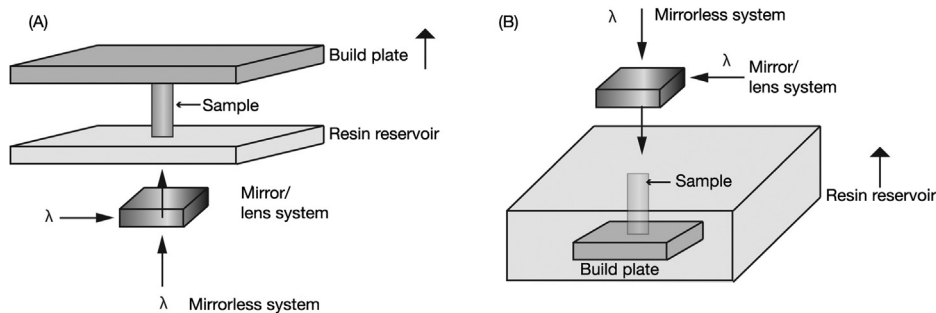


FIGURE 9.3

Two different stereolithography techniques are shown above. Each technique controls the Z -resolution by the use of a stepper motor that moves the build plate. The X - and Y -axis resolutions are controlled through the use of mirrors or direct illumination of each volumetric pixel (voxel). Both stereolithography techniques can utilize the same light source for photoinitiation and either print layer-by-layer or one voxel at a time; however, the direction of the build plate in relation to the printing resin is different. In the first case, (A) the resin reservoir remains stationary and the build plate moves upwards, away from the resin, as each layer is polymerized. The second case, (B) the resin reservoir moves up in height as each layer is built up from the stationary build plate. Both of these techniques can be used in the fabrication of scaffolds for vascular regeneration applications; however, there can be size limitations based upon the ability of the printed resin to adhere to the build plate. This can be problematic for printing method (A) where the adhered first layer must support the weight of the entire printed scaffold.

without the concern of scaffold detaching from the build plate during the printing process. This can be a concern in the previous printing method due to the fact that the polymer is usually cured directly onto a glass build plate and the adhesion might not be strong enough to counteract the force of gravity, especially for a large build.

This printing method is of interest for vascular engineering due to the variety of materials that are available for the printing process and the fact that many of these materials can be biodegradable, thus, as they degrade, the native tissue can invade and resorb the printed scaffold. Since either a UV or visible light source can be used in this process, photoinitiators that are commonly used, and potentially approved by the Food and Drug Administration, for the polymerization of biomaterials can be utilized. Scaffolds generated using this method of fabrication can also have their mechanical properties modulated based upon the curing time for each layer of the scaffold (Chiu et al., 2019; Melchiorri et al., 2016). This allows for the generation of scaffolds that have mechanical properties very similar to the native vasculature, which is critical for the prevention of intimal hyperplasia. However, there are some drawbacks to the use of this sort of rapid fabrication process to manufacture vascular grafts. The most important of which is the potential of the solvent remaining on or in the scaffold after the manufacturing process. This results in the need to wash the scaffold during the post-processing steps to ensure that all of the uncrosslinked monomers are removed, along with any remaining solvent. Another drawback is that support scaffolds must be generated to help hold the print in place, and as a result, these supports must be removed, which can prove to be a tedious endeavor for complex geometries and requires some additional postprocessing to obtain the desired surface finish on the printed vascular graft. However, as mentioned previously, one important and desirable trait of this printing process is that multiple grafts can be printed simultaneously and the printing speed is independent of the complexity of the implant design. For vascular grafts or implants, this feature can be seen as advantageous in the generation of patient-specific implants, i.e., every patient will have their own requirements that will vary in complexity based upon the native tissue architecture being replaced. Much like the other grafts/scaffolds generated for vascular regeneration, cell-free scaffolds generated in this manner can be modified postprinting to contain various bioactive molecules to encourage proper cell attachment and discourage an immune response.

One application that has seen some use in tissue engineering is the generation of artificial heart valves and conduits. Research groups have developed models based upon X-ray computed tomography and magnetic resonance imaging scans to determine the portions that need to be replaced to treat the patients' conditions. The 3D models were then printed layer-by-layer with stereolithography out of poly-4-hydroxybutyrate and polyhydroxyoctanoate, both of which allow for the generation of a biocompatible implant. For the replacement conduits and the trileaflet valves, these printed models were then tested in perfusion bioreactors (Sodian et al., 2002, 2005). The application of stereolithography to the printing of biocompatible polymers for vascular regeneration has the promise of providing a method that can construct intricate implants that can have multiple components within one printed model.

9.2.3 FUSED-DEPOSITION MODELING

FDM has been around for several years in tissue engineering applications (Hutmacher et al., 2001; Zein, Hutmacher, Tan, & Teoh, 2002). However, it has recently seen an increase in its usage in

both industrial as well as personal applications. In fact, systems based on the RepRap open source 3D printer have been utilized for tissue engineering applications (Miller et al., 2012).

In these systems, a material such as a thermoplastic or a glass is fed from a source coil into a heated extruder. The material is then deposited layer-by-layer, and the scaffold is created in this step-wise manner. The rate of extrusion, diameter of the nozzle head, temperature of the nozzle, and the speed of the nozzle head all influence the diameter of the deposited filament. Typically, as the speed of the nozzle increases for a given extrusion rate and nozzle size, the diameter of the fiber decreases. This can be used to tailor the size of the deposited filament such that it is smaller than the diameter of the extrusion nozzle. Furthermore, based on the temperature of the extrusion process, it is possible to control the adhesion of the extruded filament to previously deposited layers. This allows for the creation of interconnected filaments that are deposited according to the 3D design. Another method to create interconnected scaffolds with improved mechanical properties is the incorporation of thermally expandable microspheres into FDM matrix, which fill voids between deposited layers during a postprinting thermal treatment (Wang, Xie, Weng, Senthil, & Wu, 2016) (Figure 9.4).

However, for vascular engineering, the conditions for extruding these materials is not conducive for cell growth and, as a result, approaches using this method generally use 3D printing to deposit a scaffold that will ultimately be removed. The sacrificial scaffolds are extruded according to a 3D design, and the rate of extrusion as well as temperature is tightly controlled to ensure that a proper filament diameter is being deposited as the scaffold is being printed. Since a motorized stage is being used to control the deposition in the x – y directions, the resolution in these directions is typically limited to 100 μm . This is also dependent upon the material being used so the resolution can be improved depending on the application. In the case of generating scaffolding for vasculature and microvasculature, the scaffolding material can be a carbohydrate glass that is capable of being

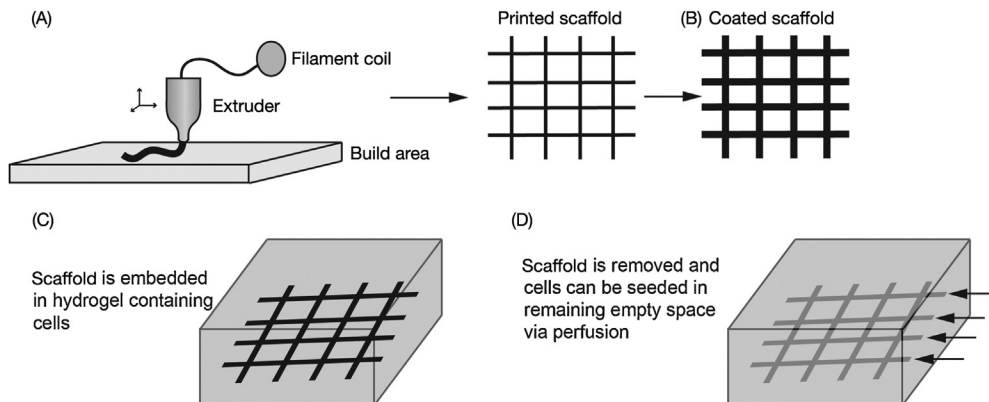


FIGURE 9.4

This illustration shows how the extruded filament is deposited creating an interconnected three-dimensional scaffold. (A) The scaffold is deposited in the desired pattern. (B) The scaffold is coated with a biocompatible polymer to protect it from the ECM/hydrogel laden with cells that is (C) deposited around the scaffolding. (D) The scaffolding is dissolved by the perfusion of water through the scaffold. ECs are injected into the interconnected network and line the walls forming vasculature/microvasculature.

dissolved postprinting without the use of harsh solvents (Miller et al., 2012). These glasses can be extruded at a controlled rate and result in filament fibers ranging from 150 μm in diameter up to the size of the filament.

This method allows for the unique ability to generate scaffolds that have vasculature as well as microvasculature. After the fabrication of the sacrificial scaffold, the scaffold is then coated with a protective layer that prevents the scaffold from degrading by the deposited matrix. This matrix can be a hydrogel with embedded cells. This hydrogel can be a wide range of gels such as alginate, fibrin, or Matrigel. Importantly, the hydrogel can be chosen based upon the desired cell type that will be encapsulated and vascularized once the scaffolding is removed. The hydrogel can be cross-linked either chemically, through the use of a photoinitiator (if the scaffold is sufficiently optically clear), or by modulating the temperature of the hydrogel plus scaffold. After the hydrogel is set with the desired cell type encapsulated, the sacrificial scaffold is then removed by perfusing water or media through the scaffold. Upon the removal of the scaffold, ECs could then be seeded onto the inner surface of the leftover porous network. This vasculature could then be placed under flow allowing for nutrient transfer from the cell culture media to the inside of the hydrogel, where typically cells would not survive due to diffusion limits and nutrient exchange.

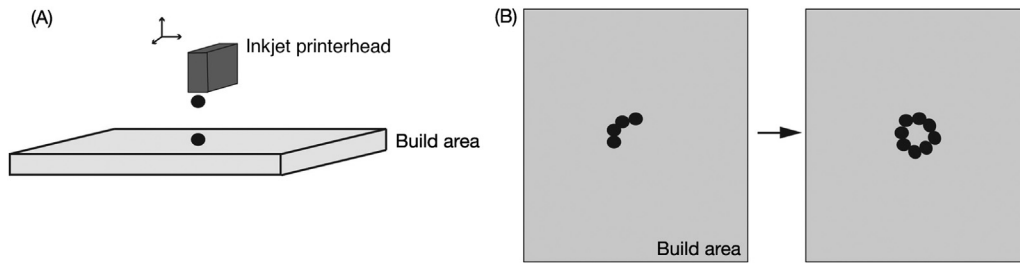
Other applications based upon this technology have been rapidly advancing (Sodian et al., 2002, 2005) and prove that cell-free methods still have the potential of providing vascularization to tissue-engineered implants. Thus allowing for the formation of larger tissue-engineered implants, while, at the same time, allowing for proper nutrient exchange ensuring viability of the implanted vascularized construct.

9.3 CELL-BASED SCAFFOLDS

Due to the ever-changing market for commercial and research-based 3D printing systems, it is very difficult to discuss the merits of individual systems for their application to a specific research problem. As such, this section will focus mainly on the technologies and concepts behind a few cell-based printing techniques that have been directly applied to the problem of vascularization and angiogenesis. This section aims to give the reader an understanding of the principles behind the different printing processes as well as a working knowledge of how the individual technologies currently compare. Three-dimensional printing of cells or cell-laden tissue-engineered implants can be broken down into a few main technologies, many of which have been around for several decades and can be further divided based upon components utilized: (1) inkjet printing, (2) extrusion-based printing, and (3) laser-assisted printing.

9.3.1 INKJET PRINTING

As one of the main, current methods for generating 3D scaffolds for tissue engineering, inkjet printing has several advantages (Figure 9.5) (Boland, Xu, Damon, & Cui, 2006; Roth et al., 2004). Due to the fact that inkjet printing technologies have been around for several decades, the cost of production of these systems is relatively low and access to the parts necessary to build a system are readily available (Billiet, Vandenhaute, Schelfhout, Van Vlierberghe, & Dubruel, 2012; Cui &

**FIGURE 9.5**

An example of inkjet printing of cells is shown. (A) Here the cells are embedded into a matrix that is compatible with an inkjet printer (piezoelectric or thermal inkjet) and printed droplet by droplet to assemble a 3D scaffold. (B) A top-down view shows how the printer head can be moved in the x – y direction laying down each droplet to form the basis of vasculature. The next layer can then be built upon these first droplets and the fabrication process continues in this iterative manner. Although this process is capable of printing scaffolds alone, the ability to print a single cell at a time at a desired location allows for the generation of heterogeneous scaffolds required for blood vessel regeneration. Additionally, the small size of the droplets allows for the fabrication of microvessels as well as regular vessels.

Boland, 2009; Cui, Boland, D’Lima, & Lotz, 2012). Inkjet printing has the ability to rapidly deposit cells and their scaffold thus enabling the rapid manufacturing of scaffolds (Nishiyama et al., 2009; Saunders, Gough, & Derby, 2008). However, this speed also has some drawbacks since cell viability can be negatively impacted if the speeds utilized in the printing process subject the cells to very high external forces; thus, material selection, viscosity, and printing speed need to be carefully selected for a given application. Additionally, obtaining high cell densities required for the long-term stability and viability of an implant is also difficult due to the printing process. Nonetheless this printing method has made significant advances in the past several years and provides a robust platform for developing functional tissues and can be used to generate scaffolds based off of images (Arai et al., 2011; Campbell & Weiss, 2007; Jakab et al., 2010; Marga et al., 2012).

One key aspect of developing functional 3D-printed tissues is the ability to utilize multiple cell types during the printing process. Specifically, it is desirable to have heterogeneous materials and ECM deposited to fabricate the new tissue-engineered implant. Inkjet printing is one of the technologies well suited to this application (Cui & Boland, 2009; Nakamura et al., 2005; Phillippi et al., 2008; Xu et al., 2005, 2006; Yamazoe & Tanabe, 2009). This is due to the ability of this method to accurately position multiple cell types and their respective matrices in precise locations of the printed scaffold. Furthermore, it has been demonstrated that this technique can be utilized to print single cells as well as heterogeneous scaffolds (Cui et al., 2012; Nakamura et al., 2005; Norotte, Marga, Niklason, & Forgacs, 2009; Xu et al., 2005). In addition to printing hydrogels laden with cells, this technology can print liquids containing cells such as alginate directly into a crosslinking solution such as calcium chloride (Christensen et al., 2015; Song et al., 2011; Xu, Chai, Huang, & Markwald, 2012).

Although there are commercial options available for inkjet printing, multiple research groups have developed their own printers based off of commercial, off-the-shelf inkjet printers. These printers need to be modified to print in the z -direction and the cartridges need to be modified and sterilized for use with cells in a sterile environment. Typically, for vascular applications, collagen-based

composites are utilized due to collagen's ability to encourage vasculogenesis *in vitro* but other materials have been used for blood vessel regeneration (Billiet et al., 2012; Fedorovich et al., 2007; Malda et al., 2013; Nishiyama et al., 2009). For microvascular applications, materials such as fibrin have been utilized for the fabrication process (Cui & Boland, 2009).

This method has been successfully used to produce constructs that mimic native vasculature as well as in the printing of heterogeneous cell populations to encourage the vascularization of the printed biomaterial. Upon implantation into a mouse model, the ability of a printed material laden with multiple cell types to encourage vasculogenesis *in vivo* has been shown. As such, this method of producing tissue-engineered blood vessels has been steadily making forward progress from *in vitro* studies to *in vivo* with the end goal of translating to clinical applications.

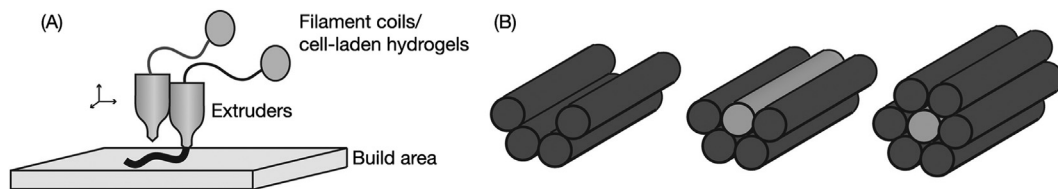
9.3.2 EXTRUSION-BASED BIOPRINTING

Mechanical extruders provide another method of generating 3D scaffolds with cells embedded. In this approach, there are advantages due to the fact that each deposited bead can be thought of as a tissue fragment. However, this method is currently expensive due to the upfront cost of the printers and the technology required. The basic concept behind this printing method is that droplets (multi-cellular particles + desired scaffold material) are deposited on the template at desired locations based upon a computer-generated design.

The building blocks used in this method, much like those used for the other printing techniques, can contain either a single cell type or a heterogeneous population. For the generation of vascular tissue, however, it is important that individual cell types are used to ensure proper layering and spatial arrangement in the final printed scaffold. Another consideration that is important for the success of these implants is that the final printed construct must be as similar as possible, structurally and functionally, to the native tissue.

As such, materials such as collagen hydrogels have been successfully used as scaffolds to encapsulate the cells during the printing process. There are potentially several drawbacks depending on the chosen printing parameters. For example, the system may place the hydrogels under high pressure during the printing process. Based on the viscosity of the printed hydrogel, the shear force exerted on the cells may be of large enough magnitude to cause cell death. Additionally, this method of printing is currently restricted to a very specific set of materials; thus, limiting the number of overall applications. However, by careful selection of materials and engineering it has been possible to produce 3D-printed tissues that mimic the native vasculature in terms of distinct functional layers with the ability to apply flow to the inner channel.

Figure 9.6 illustrates how extrusion bioprinting can be applied to the production of tissue-engineered vasculature. The layers are built in a stepwise manner where the outermost layers are deposited with cells embedded. The inner layer, which will have the applied flow once the construct is complete, needs to be printed with an additional material that can be removed in the final printed product without compromising the seeded cell viability. Pluronic F-127 (Kang et al., 2016; Kolesky et al., 2014; Kolesky, Homan, Skylar-Scott, & Lewis, 2016) and sodium alginate (Jia et al., 2016) are commonly used as the sacrificial material for vascularized tissue constructs. Another example of a secondary material that can be used for this process is agarose. The printed agarose rods fill in the void spaces during the printing process, which allow for the deposition of layers above the desired void space and prevent cell invasion and remodeling (Jakab et al., 2010).

**FIGURE 9.6**

The basic setup of an extrusion-based 3D printer is shown above in (A). The printer consists of a build plate and a printer head/nozzle on a motorized axis. The printing nozzle can contain either a polymer for extrusion or a biomaterial loaded with cells, and it moves in the X, Y, and Z directions depositing each layer in the desired location. These layers can be built off of each other much like the other 3D printing processes. However, one distinction is that the extrusion rate and the speed of the nozzle can be used to modulate the fiber size being deposited. In the case of printing vasculature, void spaces can either be included by not depositing any material or by the printing of a second material that provides structural support during the printing process. An example of this is shown in (B) where the desired cell-laden scaffold is built layer-by-layer with one nozzle (black cylinders) and a second nozzle containing a secondary material (gray cylinder) that is used to deposit a support structure to be removed after the printing process is complete. A 3D vasculature can be fabricated in this manner with a controlled vasculature shape and inner diameter once the secondary matrix is removed (gray cylinder).

Adapted from Jakab et al. (2010)

After completion of the printing process and the final gelation of the printed scaffold, the agarose rods could be removed from the center of the print. Thus the printed scaffold has defined layers and an inner void that allows for the flow of nutrients/media as well as separate functional layers. For vascular applications, this dual print method is especially important due to the weak mechanical properties typically associated with the hydrogels compatible with this printing process.

An additional application of this printing method for vascular regeneration was in the fabrication of more complex structures such as the printing of an aortic valve. In this work, researchers successfully fabricated a 3D scaffold laden with different cell types and maintained viability for 1 week (Ghista & Kabinejadian, 2013). This research demonstrates the ability to apply this technology to the various structures that comprise the vascular system. As such, this method possesses great promise in addressing the needs for tissue-engineered vasculature and in aiding blood vessel regeneration.

9.3.2.1 Coaxial Printing

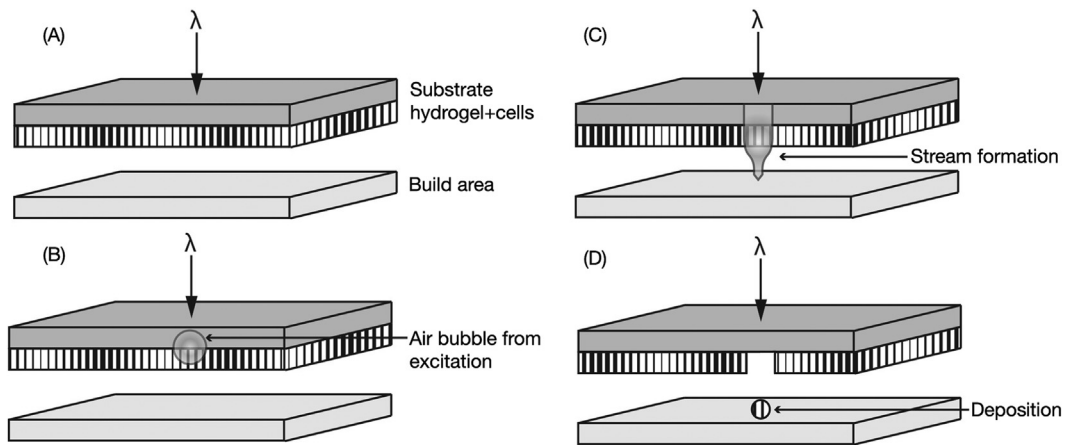
Coaxial printing is an emerging technology for building vascular conduits, as well as vascularized tissues. This method allows the preservation of lumen structures in vascular networks through self-supported printing with a needle composed of multiple concentric rings that create separate chambers for multilayer extrusion printing. In some instances, the outer ring is used to extrude cell-loaded sodium alginate, while the inner ring supplies CaCl_2 for crosslinking (Zhang et al., 2015). Other methods utilize gelatin (Shao et al., 2020) or Pluronic F-127 (Gao et al., 2017, 2019; Gao, Park, Kim, Jang, & Cho, 2018) as the sacrificial material to support the printing of ECs, endothelial progenitor cells, SMCs, and other critical vascular components. Triple-coaxial printing was implemented in a bilayered vascular graft to recapitulate the tunica intima and the tunica media layers, which remained patent over 3 weeks as a rat interpositional abdominal aorta graft (Gao et al., 2019).

9.3.3 LASER-ASSISTED PRINTING

One of the potential problems associated with the 3D printing of cells is due to the limited cell densities possible based upon the printing technique chosen. Laser-assisted cell printing can overcome the low viability and low cell density problem (Guillotin et al., 2010; Koch, Gruene, Unger, & Chichkov, 2013; Tasoglu & Demirci, 2013). The technique of laser printing is not new, as it has been used in the fabrication of circuits and biosensors. However, its application to layer-by-layer cell deposition is relatively recent and allows for easy control of cell density in the final printed product. This method overcomes limitations on cell seeding density by seeding a source gel at a high density and ensuring the cells' viability before the printing process (Barron et al., 2004; Barron, Krizman, & Ringeisen, 2005; Guillotin et al., 2010). The source gel is transferred to a target or collection slide on a spot-by-spot basis through the excitation of the gel or a specialized substrate. There are currently two main modes of excitation where either the excitation energy is directly matched by the continuous wave laser source or a two-photon, pulsed excitation source is used, where the required excitation energy is approximately double the laser energy.

There are many pros and cons of using two-photon excitation; however, the most important of which are the ability to focus the excitation energy to very small volumes and the large upfront cost of the system especially with a tunable excitation source. Although there has been some success with Nd:YAG and Ti:sapphire tunable lasers, the costs of these systems can be prohibitive for the development of a two-photon printing system for tissue engineering applications. Even though there are differences in terms of the generation of the required excitation energy, the basic concept and procedure of using continuous wave and pulsed lasers for laser-assisted cell printing remains basically the same. Cells before the printing process can be cultured normally and trypsinized to remove them from their culturing environment. Upon resuspension in a compatible hydrogel (collagen, alginate, fibrin, etc.), the hydrogel is mounted on a glass slide with or without a coating of a laser radiation absorbing layer. This glass slide is then positioned parallel to a collection slide several hundred micrometers to a few millimeters away. The basic principle of this printing process is that an excitation source is focused on a small point above the desired deposition location. The focused light causes the local expansion of gases (through the evaporation of either the laser radiation absorbing layer or the hydrogel directly) which then provides the kinetic energy required to push the hydrogel beneath the focal point toward the collection slide. The exposure time/intensity of the laser can adjust the volume ejected from hydrogel, and as such, volumes can be adjusted on the order of picoliters, allowing for very rigorous control of the ejected volume and cell seeding density of the printed construct. The spatial resolution of this technique also has some unique advantages due to the ability to precisely control the focal point of excitation, controlling the ejection of hydrogel loaded with cells from the source plate toward the collection plate (Figure 9.7).

Additionally, the properties of the ejected material can be tailored to the application at hand. Depending on the viscosity of the source hydrogel, either a droplet or a stream can be formed upon excitation by the light source. This in turn can help to control the shape of the patterns deposited on the collection plate. In the case of vascular regeneration, this technique has a unique advantage because multiple source plates with different cell types embedded in different hydrogels can be used to build the vascular implant. The ECs could be deposited along an inner ring with SMCs surrounding on the outside and based upon the implant location, the diameter of the scaffold and the thickness of the layers can be adjusted to mimic the relative ratios present in the native vasculature.

**FIGURE 9.7**

A typical setup for laser-assisted bioprinting is shown in the above illustration. (A) The excitation source is focused on a point on the glass slide containing the hydrogel with cells embedded. (B) An air bubble forms from the excitation of the substrate. (C) The air bubble then can lead to a stream formation that propels the cells + matrix toward the build area. (D) The build area is positioned microns to millimeters away to serve as a collection slide for the droplets produced from the rapid expansion of the radiation absorbing layer. In the case of blood vessel regeneration, this technique has a few drawbacks in regard to the mechanical strength of the printed structure, but it excels in regard to spatial resolution as well as the ability to exchange the donor plate with another with a separate matrix and cellular composition. As such, it can print heterogeneous scaffolds that have the potential for use in the creation of vasculature as well as microvasculature. This sort of additive manufacturing has been used to generate cell-based scaffolds to mimic the native vasculature.

These concentric rings can be printed one on top of another thereby building up complex architecture in a layer-by-layer approach.

One drawback to the utilization of this method for vascular regeneration is that hydrogels can have very different mechanical properties from the native tissue. Even though the final printed construct will only have the mechanical properties of the hydrogels used, which tend to be too weak on their own without additional support, the cells deposited can be precisely deposited in heterogeneous layers that very closely mimics the native heterogeneous properties of vasculature. Furthermore, these cells will be positioned close to one another such that appropriate cell–cell adhesions and communications can be established thus enabling the printed scaffold to develop into a functioning implant. Also, since this method can precisely deposit very small volumes, it may be possible to use this fabrication approach to build microvasculature that has a very small inner diameter and very few cells are needed to create each of the concentric layers that comprise the vasculature.

Although laser-assisted 3D printing has been successfully used to create tissue-engineered blood vessels, it also has been used for patterning stem cells and ECs for cardiac regeneration (Gaebel et al., 2011). Additionally, this technology has been utilized for complex tissues such as bioprinting skin with its various ECM and cellular components (Koch et al., 2012; Pirlo, Wu, Liu, & Ringeisen, 2012).

9.4 COMPARISON OF THE TECHNOLOGIES

All of these technologies presented here represent a subset of approaches for the regeneration of vasculature. Depending on the technique used and the materials available, surface modification with proteins, cytokines, and growth factors can be included in the manufacturing process. These modifications can further enhance the cellular response to improve blood vessel regeneration. This is especially important to recruit ECs to the tunica intima of the implant to aid in the prevention of stenosis (Avci-Adali, Ziemer, & Wendel, 2010; Avci-Adali et al., 2013; Han et al., 2013; Hashi et al., 2010; He et al., 2005; Zhang et al., 2013). Additionally, the mechanical properties of the vascular implant can be modified by the recruitment or incorporation of SMCs and appropriate ECM proteins such as elastin (Berglund, Nerem, & Sambanis, 2004; Cui & Boland, 2009; Fu et al., 2014; Greenwald & Berry, 2000; Heydarkhan-Hagvall et al., 2006; Kasalkova et al., 2014; Nerem, 2003; Norotte et al., 2009; Patel, Fine, Sandig, & Mequanint, 2006; Wang, Kibbe, & Ameer, 2013). It is critical that these competing technologies incorporate the heterogeneous nature of each layer in the vasculature in the design and fabrication of scaffolds for therapeutic applications.

Although it has been shown that electrospinning can have control over each layer's orientation and fiber size (Hashi et al., 2010; He et al., 2005; Shapira et al., 2014; Subbiah et al., 2005; Wu et al., 2010; Zhang et al., 2013; Ziabari et al., 2010), these implants do not have as stringent control over each layer as rapid fabrication techniques. As the cost continues to decrease for 3D printing, the use of 3D printing to develop scaffolds for blood vessel regeneration will only increase. As previously mentioned, cell-free scaffolds tend to be modified to increase their biological activity, whereas cell-based scaffolds are already using materials that will encourage the growth of cells in substrates specific to the desired cell type. This is an important advantage and distinction because the cells are ultimately responsible for the long-term viability of the implant as well as its long-term mechanical properties. However, there is a distinct disadvantage currently with cell-based scaffolds, and that is that the base materials used have very poor mechanical properties when compared with that of native tissue or the cell-free scaffolds.

In addition, for vascular applications there are design parameters that need to be taken into account when choosing an appropriate fabrication method. For example, the complexity of the desired product in terms of surface features is very important. If only a simple cylinder is needed, then all of the above fabrication processes could be used; however, if complex overhangs or surface topologies are needed, then methods such as stereolithography and extrusion will need to be used based upon their ability to fabricate free standing structures. The desired resolution of the printing process might also be an important parameter for more complex geometries. Table 9.1 shows typical resolutions for the discussed printing techniques.

9.4.1 APPLICATIONS TO THE VASCULAR SYSTEM AND OTHER TISSUE-ENGINEERED IMPLANTS

Three-dimensional printing of the vascular system opens a wide range of applications to tissue engineering and regenerative medicine. As discussed previously, vascularization is a limiting factor in implant design and success. Additionally, complex geometries native to the vascular system could not always be replicated on the surgical table. As such, 3D printing of custom tailored

Table 9.1 Comparison of various printing technologies commonly utilized in vascular applications.

Technique	Typical resolution (μm)	Material	References
Stereolithography	25–50	Hydrogels and polymers	Billiet et al. (2012), Sodian et al. (2002, 2005)
Fused-deposition modeling	~ 100	Polymers	Miller et al. (2012), Zein et al. (2002)
Inkjet bioprinting	20 (picoliter droplets)	Liquids and hydrogels	Arai et al. (2011), Boland et al. (2006), Campbell and Weiss (2007), Cui and Boland (2009), Cui et al. (2012), Nakamura et al. (2005)
Extrusion-based bioprinting	~ 100	Hydrogels (natural and synthetic), polymers	Jakab et al. (2010), Marga et al. (2012)
Laser-assisted bioprinting	10	Hydrogels	Barron et al. (2004), Barron et al. (2005), Gaebel et al. (2011), Guillotin et al. (2010), Koch et al. (2012), Koch et al. (2013)

implants could fulfill this need by providing surgeons a means to develop implants using off-the-shelf 3D imaging techniques such as computerized tomography and magnetic resonance imaging. From these scans, complex architecture scaffolds could be developed and printed to meet the desired application and treat the underlying condition.

There are still challenges associated with these sorts of implants, ranging from cell source, cell viability, surface properties, mechanical properties, and a wide range of regulatory constraints that must be overcome before the application of these technologies to therapeutic applications. Investigations utilizing these methods, both cell-free scaffolds and cell-laden scaffolds, will yield the necessary insight into some of these problems. Furthermore, the developed materials and technologies must progress to have the desired mechanical and surface properties while at the same time steps must be taken to address the necessary regulatory requirements for clinical applications. As such, these technologies stand poised to provide insight into the direction that future research must follow to successfully develop 3D-printed vasculature for therapeutic applications.

9.5 FUTURE DIRECTIONS

This research is not just limited to simple replacement of existing vascular tissue. It also has a much broader impact on the vascularization of 3D-printed implants discussed throughout this book. All of these tissues require integration into the vascular system and by understanding the complex process of angiogenesis and developing mimetic tissue that can provide sufficient nutrient exchange. These artificial tissues can be further developed to make a significant impact on the field of tissue engineering and regenerative medicine.

Again, the predominant challenge that needs to be addressed for 3D printing of vasculature and its application to tissue-engineered constructs is the heterogeneous nature of the desired tissues. It is important that the printed implants not only mimic the natural shape of the organs and tissues but also the implants recapitulate the complex, heterogeneous nature of those tissues. For vascular implants, future directions need to address the need to have distinct layers with different mechanical properties to best mimic the native vasculature. Cell-laden constructs need to take this one step further by addressing the heterogeneous mechanical properties as well as scaffold materials for the different cell types being incorporated into the vascular implant.

The future of 3D-printed blood vessel implants will need to address the aforementioned challenges as well improve upon the rapid fabrication techniques such that the technologies developed in a laboratory setting can be translated into clinical settings by allowing for the scale up required for mass production. As such, the next several years will see advances in the materials used in the 3D printing application and in the ease of preparation of these materials for use with the various 3D printing technologies. Additionally, the use of stem cells, either obtained directly from the patient or induced from other patient cells, will have a significant impact on the design of blood vessel implants. These stem cells can be used to determine the best material environment for *in vitro* and *in vivo* population of the blood vessel implants, which will give additional insight into the critical parameters for the design of biomimetic niches within the 3D-printed implants.

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References

- Abdal-Hay, A., Bartnikowski, M., Hamlet, S., & Ivanovski, S. (2018). Electrospun biphasic tubular scaffold with enhanced mechanical properties for vascular tissue engineering. *Materials Science & Engineering. C, Materials for Biological Applications*, 82, 10–18.
- Almeida Hde, A., & Bartolo, P. J. (2010). Virtual topological optimisation of scaffolds for rapid prototyping. *Medical Engineering & Physics*, 32, 775–782.
- Arai, K., Iwanaga, S., Toda, H., Genci, C., Nishiyama, Y., & Nakamura, M. (2011). Three-dimensional inkjet biofabrication based on designed images. *Biofabrication*, 3, 1758–5082.
- Avci-Adali, M., Stoll, H., Wilhelm, N., Perle, N., Schlensak, C., & Wendel, H. P. (2013). In vivo tissue engineering: Mimicry of homing factors for self-endothelialization of blood-contacting materials. *Pathobiology: Journal of Immunopathology, Molecular and Cellular Biology*, 80, 176–181.

- Avci-Adali, M., Ziemer, G., & Wendel, H. P. (2010). Induction of EPC homing on biofunctionalized vascular grafts for rapid in vivo self-endothelialization—A review of current strategies. *Biotechnology Advances*, 28, 119–129.
- Barron, J. A., Krizman, D. B., & Ringeisen, B. R. (2005). Laser printing of single cells: Statistical analysis, cell viability, and stress. *Annals of Biomedical Engineering*, 33, 121–130.
- Barron, J. A., Wu, P., Ladouceur, H. D., & Ringeisen, B. R. (2004). Biological laser printing: A novel technique for creating heterogeneous 3-dimensional cell patterns. *Biomedical Microdevices*, 6, 139–147.
- Benjamin, E. J., Blaha, M. J., Chiuve, S. E., Cushman, M., Das, S. R., Deo, R., . . . Muntner, P. (2017). Heart disease and stroke statistics – 2017 update: A report from the American Heart Association. *Circulation*, 135, e146–e603.
- Berglund, J. D., Nerem, R. M., & Sambanis, A. (2004). Incorporation of intact elastin scaffolds in tissue-engineered collagen-based vascular grafts. *Tissue Engineering*, 10, 1526–1535.
- Billiet, T., Vandenhaute, M., Schelfhout, J., Van Vlierberghe, S., & Dubrue, P. (2012). A review of trends and limitations in hydrogel-rapid prototyping for tissue engineering. *Biomaterials*, 33, 6020–6041.
- Boland, T., Xu, T., Damon, B., & Cui, X. (2006). Application of inkjet printing to tissue engineering. *Biotechnology Journal*, 1, 910–917.
- Campbell, P. G., & Weiss, L. E. (2007). Tissue engineering with the aid of inkjet printers. *Expert Opinion on Biological Therapy*, 7, 1123–1127.
- Chiu, Y. C., Shen, Y. F., Lee, A. K. X., Lin, S. H., Wu, Y. C., & Chen, Y. W. (2019). 3D printing of amino resin-based photosensitive materials on multi-parameter optimization design for vascular engineering applications. *Polymers (Basel)*, 11, 1–13.
- Christensen, K., Xu, C., Chai, W., Zhang, Z., Fu, J., & Huang, Y. (2015). Freeform inkjet printing of cellular structures with bifurcations. *Biotechnology and Bioengineering*, 112, 1047–1055.
- Cleveland, D. C., Williams, W. G., Razzouk, A. J., Trusler, G. A., Rebeyka, I. M., Duffy, L., . . . Freedom, R. M. (1992). Failure of cryopreserved homograft valved conduits in the pulmonary circulation. *Circulation*, 86, 150–153.
- Creager, M., Loscalzo, J., & Beckman, J. A. (2012). *Vascular medicine: A companion to Braunwald's heart disease*. Elsevier Health Sciences.
- Cui, X., & Boland, T. (2009). Human microvasculature fabrication using thermal inkjet printing technology. *Biomaterials*, 30, 6221–6227.
- Cui, X., Boland, T., D'Lima, D. D., & Lotz, M. K. (2012). Thermal inkjet printing in tissue engineering and regenerative medicine. *Recent Patents on Drug Delivery & Formulation*, 6, 149–155.
- Dauphinee, S., & Karsan, A. (2010). *Endothelial dysfunction and inflammation*. Springer Basel AG.
- Deitzel, J. M., Kleinmeyer, J., Harris, D., & Beck Tan, N. C. (2001). The effect of processing variables on the morphology of electrospun nanofibers and textiles. *Polymer*, 42, 261–272.
- Derby, B. (2012). Printing and prototyping of tissues and scaffolds. *Science (New York, N.Y.)*, 338, 921–926.
- Dong, X., Yuan, X., Wang, L., Liu, J., Midgley, A. C., Wang, Z., . . . Kong, D. (2018). Construction of a bilayered vascular graft with smooth internal surface for improved hemocompatibility and endothelial cell monolayer formation. *Biomaterials*, 181, 1–14.
- Fedorovich, N. E., Alblas, J., de Wijn, J. R., Hennink, W. E., Verbout, A. J., & Dhert, W. J. A. (2007). Hydrogels as extracellular matrices for skeletal tissue engineering: State-of-the-art and novel application in organ printing. *Tissue Engineering*, 13, 1905–1925.
- Fernández-Colino, A., Wolf, F., Rütten, S., Schmitz-Rode, T., Rodríguez-Cabello, J. C., Jockenhoevel, S., & Mela, P. (2019). Small caliber compliant vascular grafts based on elastin-like recombinamers for in situ tissue engineering. *Frontiers in Bioengineering and Biotechnology*, 7, 1–13.
- Fridrikh, S. V., Yu, J. H., Brenner, M. P., & Rutledge, G. C. (2003). Controlling the fiber diameter during electrospinning. *Physical Review Letters*, 90, 144502.

- Fu, W., Liu, Z., Feng, B., Hu, R., He, X., Wang, H., ... Wang, W. (2014). Electrospun gelatin/PCL and collagen/PLCL scaffolds for vascular tissue engineering. *International Journal of Nanomedicine*, 9, 2335–2344.
- Gaebel, R., Ma, N., Liu, J., Guan, J., Koch, L., Klopsch, C., ... Steinhoff, G. (2011). Patterning human stem cells and endothelial cells with laser printing for cardiac regeneration. *Biomaterials*, 32, 9218–9230.
- Gao, G., Kim, H., Kim, B. S., Kong, J. S., Lee, J. Y., Park, B. W., ... Cho, D. W. (2019). Tissue-engineering of vascular grafts containing endothelium and smooth-muscle using triple-coaxial cell printing. *Applied Physics Reviews*, 6, 041402.
- Gao, G., Lee, J. H., Jang, J., Lee, D. H., Kong, J. S., Kim, B. S., ... Cho, D. W. (2017). Tissue engineered bio-blood-vessels constructed using a tissue-specific bioink and 3D coaxial cell printing technique: A novel therapy for ischemic disease. *Advanced Functional Materials*, 27, 1–12.
- Gao, G., Park, J. Y., Kim, B. S., Jang, J., & Cho, D. W. (2018). Coaxial cell printing of freestanding, perfusable, and functional in vitro vascular models for recapitulation of native vascular endothelium pathophysiology. *Advanced Healthcare Materials*, 7, 1–12.
- Gao, Y., Yi, T., Shinoka, T., Lee, Y. U., Reneker, D. H., Breuer, C. K., & Becker, M. L. (2016). Pilot mouse study of 1 mm inner diameter (ID) vascular graft using electrospun poly(ester urea) nanofibers. *Advanced Healthcare Materials*, 5, 2427–2436.
- Ghista, D. N., & Kabinejadian, F. (2013). Coronary artery bypass grafting hemodynamics and anastomosis design: A biomedical engineering review. *Biomedical Engineering Online*, 12, 12–129.
- Go, A. S., Mozaffarian, D., Roger, V. L., Benjamin, E. J., Berry, J. D., Borden, W. B., ... Turner, M. B. (2012). Heart disease and stroke statistics—2013 update: A report from the American Heart Association. *Circulation*, 127, e6–e245.
- Gong, W., Lei, D., Li, S., Huang, P., Qi, Q., Sun, Y., ... Zhao, Q. (2016). Hybrid small-diameter vascular grafts: Anti-expansion effect of electrospun poly ϵ -caprolactone on heparin-coated decellularized matrices. *Biomaterials*, 76, 359–370.
- Greenwald, S. E., & Berry, C. L. (2000). Improving vascular grafts: The importance of mechanical and haemodynamic properties. *The Journal of Pathology*, 190, 292–299.
- Guillotin, B., Souquet, A., Catros, S., Duocastella, M., Pippenger, B., Bellance, S., ... Guillemot, F. (2010). Laser assisted bioprinting of engineered tissue with high cell density and microscale organization. *Biomaterials*, 31, 7250–7256.
- Hadjizadeh, A., Ajji, A., Jolicoeur, M., Liberelle, B., & De Crescenzo, G. (2013). Effects of electrospun nanostructure vs microstructure on human aortic endothelial cell behavior. *Journal of Biomedical Nanotechnology*, 9, 1195–1209.
- Han, F., Jia, X., Dai, D., Yang, X., Zhao, J., Zhao, Y., ... Yuan, X. (2013). Performance of a multilayered small-diameter vascular scaffold dual-loaded with VEGF and PDGF. *Biomaterials*, 34, 7302–7313.
- Hashi, C. K., Derugin, N., Janairo, R. R. R., Lee, R., Schultz, D., Lotz, J., & Li, S. (2010). Antithrombogenic modification of small-diameter microfibrillar vascular grafts. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 30, 1621–1627.
- He, S., Xia, T., Wang, H., Wei, L., Luo, X., & Li, X. (2012). Multiple release of polyplexes of plasmids VEGF and bFGF from electrospun fibrous scaffolds towards regeneration of mature blood vessels. *Acta Biomaterialia*, 8, 2659–2669.
- He, W., Yong, T., Teo, W. E., Ma, Z., & Ramakrishna, S. (2005). Fabrication and endothelialization of collagen-blended biodegradable polymer nanofibers: Potential vascular graft for blood vessel tissue engineering. *Tissue Engineering*, 11, 1574–1588.
- Heydarkhan-Hagvall, S., Esguerra, M., Helenius, G., Soderberg, R., Johansson, B. R., & Risberg, B. (2006). Production of extracellular matrix components in tissue-engineered blood vessels. *Tissue Engineering*, 12, 831–842.

- Hirt, M. N., Hansen, A., & Eschenhagen, T. (2014). Cardiac tissue engineering: State of the art. *Circulation Research*, 114, 354–367.
- Horn, T. J., & Harrysson, O. L. (2012). Overview of current additive manufacturing technologies and selected applications. *Science Progress*, 95, 255–282.
- Huang, R., Gao, X., Wang, J., Chen, H., Tong, C., Tan, Y., & Tan, Z. (2018). Triple-layer vascular grafts fabricated by combined E-Jet 3D printing and electrospinning. *Annals of Biomedical Engineering*, 46, 1254–1266.
- Hutmacher, D. W., Schantz, T., Zein, I., Ng, K. W., Teoh, S. H., & Tan, K. C. (2001). Mechanical properties and cell cultural response of polycaprolactone scaffolds designed and fabricated via fused deposition modeling. *Journal of Biomedical Materials Research*, 55, 203–216.
- Iaizzo, P.A. (2005). Handbook of cardiac anatomy, physiology, and devices. Humana Press Inc. <https://doi.org/10.1007/978-1-59259-835-9>.
- Jakab, K., Norotte, C., Marga, F., Murphy, K., Vunjak-Novakovic, G., & Forgacs, G. (2010). Tissue engineering by self-assembly and bio-printing of living cells. *Biofabrication*, 2, 1758–5082.
- Jia, W., Gungor-Ozkerim, P. S., Zhang, Y. S., Yue, K., Zhu, K., Lio, W., ... Khademhosseini, A. (2016). Direct 3D bioprinting of perfusable vascular constructs using a blend bioink. *Biomaterials*, 106, 58–68.
- Jonas, R. A., Freed, M. D., Mayer, J. E., & Castaneda, A. R. (1985). Long-term follow-up of patients with synthetic right heart conduits. *Circulation*, 72, 77–83.
- Ju, Y. M., Ahn, H., Arenas-Herrera, J., Kim, C., Abolbashari, M., Atala, A., ... Lee, S. J. (2017). Electrospun vascular scaffold for cellularized small diameter blood vessels: A preclinical large animal study. *Acta Biomaterialia*, 59, 58–67.
- Junqueira, L., & Carneiro, J. (1995). *Basic histology*. Appleton & Lange.
- Kang, H., Lee, S. J., Ko, I. K., Kengla, C., Yoo, J. J., & Atala, A. (2016). A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. *Nature Biotechnology*, 34, 312–322.
- Kasálková, N. S., Slepíčka, P., Kolská, Z., Hodačová, P., Kučková, S., & Svorčík, V. (2014). Grafting of bovine serum albumin proteins on plasma-modified polymers for potential application in tissue engineering. *Nanoscale Research Letters*, 9, 161.
- Koch, L., Deiwick, A., Schlie, S., Michael, S., Gruene, M., Coger, V., ... Chichkov, B. (2012). Skin tissue generation by laser cell printing. *Biotechnology and Bioengineering*, 109, 1855–1863.
- Koch, L., Gruene, M., Unger, C., & Chichkov, B. (2013). Laser assisted cell printing. *Current Pharmaceutical Biotechnology*, 14, 91–97.
- Kolesky, D. B., Homan, K. A., Skylar-Scott, M. A., & Lewis, J. A. (2016). Three-dimensional bioprinting of thick vascularized tissues. *Proceedings of the National Academy of Sciences of the United States of America*, 113, 3179–3184.
- Kolesky, D. B., Truby, R. L., Gladman, A. S., Busbee, T. A., Homan, K. A., & Lewis, J. A. (2014). 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. *Advanced Materials*, 26, 3124–3130.
- Kozel, B. A., Mecham, R. P., & Rosenbloom, J. (2011). *The extracellular matrix: An overview*. Berlin: Springer-Verlag. Available from http://doi.org/10.1007/978-3-642-16555-9_8.
- Lai, H. J., Kuan, C. H., Wu, H. C., Tsai, J. C., Chen, T. M., Hsieh, D. J., & Wang, T. W. (2014). Tailored design of electrospun composite nanofibers with staged release of multiple angiogenic growth factors for chronic wound healing. *Acta Biomaterialia*, 9, 204–209.
- Lamers, L. J., Frommelt, P. C., Mussatto, K. A., Jaquiss, R. D. B., Mitchell, M. E., & Tweddell, J. S. (2012). Coarctectomy combined with an interdigitating arch reconstruction results in a lower incidence of recurrent arch obstruction after the Norwood procedure than coarctectomy alone. *The Journal of Thoracic and Cardiovascular Surgery*, 143, 1098–1102.
- Li, Z., Zhou, P., Zhou, F., Zhao, Y., Ren, L., & Yuan, X. (2018). Antimicrobial eugenol-loaded electrospun membranes of poly(ϵ -caprolactone)/gelatin incorporated with REDV for vascular graft applications. *Colloids Surfaces B Biointerfaces*, 162, 335–344.

- Luong-Van, E., Grondahl, L., Chua, K. N., Leong, K. W., Nurcombe, V., & Cool, S. M. (2006). Controlled release of heparin from poly(epsilon-caprolactone) electrospun fibers. *Biomaterials*, 27, 2042–2050.
- Malda, J., Visser, J., Melchels, F. P., Jüngst, T., Hennink, W. E., Dhert, W. J. A., ... Huttmacher, D. W. (2013). 25th Anniversary article: Engineering hydrogels for biofabrication. *Advanced Materials*, 25, 5011–5028.
- Marga, F., Jakab, K., Khatiwala, C., Shepherd, B., Dorfman, S., Hubbard, B., ... Gabor, F. (2012). Toward engineering functional organ modules by additive manufacturing. *Biofabrication*, 4, 1758–5082.
- Melchels, F. P. W., Domingos, M. A. N., Klein, T. J., Malda, J., Bartolo, P. J., & Huttmacher, D. W. (2012). Additive manufacturing of tissues and organs. *Progress in Polymer Science*, 37, 1079–1104.
- Melchiorri, A. J., Hibino, N., Best, C. A., Yi, T., Lee, Y. U., Kraynak, C. A., ... Fisher, J. P. (2016). 3D-printed biodegradable polymeric vascular grafts. *Advanced Healthcare Materials*, 5, 319–325.
- Miller, J. S., Stevens, K. R., Yang, M. T., Baker, B. M., Nguyen, D. H., Cohen, D. M., ... Chen, C. S. (2012). Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues. *Nature Materials*, 11, 768–774.
- Montero, R. B., Vial, X., Nguyen, D. T., Farhand, S., Reardon, M., Pham, S. M., ... Andreopoulos, F. M. (2012). bFGF-containing electrospun gelatin scaffolds with controlled nano-architectural features for directed angiogenesis. *Acta Biomaterialia*, 8, 1778–1791.
- Montini-Ballarín, F., Calvo, D., Caracciolo, P. C., Rojo, F., Frontini, P. M., Abraham, G. A., & Guinea, G. V. (2016). Mechanical behavior of bilayered small-diameter nanofibrous structures as biomimetic vascular grafts. *Journal of the Mechanical Behavior of Biomedical Materials*, 60, 220–233.
- Nakamura, M., Kobayashi, A., Takagi, F., Watanabe, A., Hiruma, Y., Ohuchi, K., ... Takatani, S. (2005). Biocompatible inkjet printing technique for designed seeding of individual living cells. *Tissue Engineering*, 11, 1658–1666.
- Nerem, R. M. (2003). Role of mechanics in vascular tissue engineering. *Biorheology*, 40, 281–287.
- Nishiyama, Y., Nakamura, M., Henmi, C., Yamaguchi, K., Mochizuki, S., Nakagawa, H., & Takiura, K. (2009). Development of a three-dimensional bioprinter: Construction of cell supporting structures using hydrogel and state-of-the-art inkjet technology. *Journal of Biomechanical Engineering*, 131, 3002759.
- Norotte, C., Marga, F. S., Niklason, L. E., & Forgacs, G. (2009). Scaffold-free vascular tissue engineering using bioprinting. *Biomaterials*, 30, 5910–5917.
- Norouzi, S. K., & Shamloo, A. (2019). Bilayered heparinized vascular graft fabricated by combining electrospinning and freeze drying methods. *Materials Science & Engineering. C, Materials for Biological Applications*, 94, 1067–1076.
- Patel, A., Fine, B., Sandig, M., & Mequanint, K. (2006). Elastin biosynthesis: The missing link in tissue-engineered blood vessels. *Cardiovascular Research*, 71, 40–49.
- Phillippi, J. A., Miller, E., Weiss, L., Huard, J., Waggoner, A., & Campbell, P. (2008). Microenvironments engineered by inkjet bioprinting spatially direct adult stem cells toward muscle- and bone-like subpopulations. *Stem Cells*, 26, 127–134.
- Pirlo, R. K., Wu, P., Liu, J., & Ringeisen, B. (2012). PLGA/hydrogel biopapers as a stackable substrate for printing HUVEC networks via BioLP. *Biotechnology and Bioengineering*, 109, 262–273.
- Qiu, X., Lee, B. L. P., Ning, X., Murthy, N., Dong, N., & Li, S. (2017). End-point immobilization of heparin on plasma-treated surface of electrospun polycarbonate-urethane vascular graft. *Acta Biomaterialia*, 51, 138–147.
- Rayatpisheh, S., Heath, D. E., Shakouri, A., Rujitanaroj, P. O., Chew, S. Y., & Chan-Park, M. B. (2014). Combining cell sheet technology and electrospun scaffolding for engineered tubular, aligned, and contractile blood vessels. *Biomaterials*, 35, 2713–2719.
- Roger, V. L., Go, A. S., Lloyd-Jones, D. M., Benjamin, E. J., Berry, J. D., Borden, W. B., ... Subcomm, S. S. (2012). Heart disease and stroke statistics—2012 update a report from the American Heart Association. *Circulation*, 125, E2–E220.

- Roth, E. A., Xu, T., Das, M., Gregory, C., Hickman, J. J., & Boland, T. (2004). Inkjet printing for high-throughput cell patterning. *Biomaterials*, 25, 3707–3715.
- Roy, K. (2010). *Biomaterials as stem cell niche*. Springer.
- Sales, V. L., Engelmayr, G. C., Mettler, B. A., Johnson, J. A., Sacks, M. S., & Mayer, J. E. (2006). Transforming growth factor-beta 1 modulates extracellular matrix production, proliferation, and apoptosis of endothelial progenitor cells in tissue-engineering scaffolds. *Circulation*, 114, I193–I199.
- Saunders, R. E., Gough, J. E., & Derby, B. (2008). Delivery of human fibroblast cells by piezoelectric drop-on-demand inkjet printing. *Biomaterials*, 29, 193–203.
- Schubert, C., van Langeveld, M. C., & Donoso, L. A. (2013). Innovations in 3D printing: A 3D overview from optics to organs. *The British Journal of Ophthalmology*. Available from <https://doi.org/10.1136/bjophthalmol-2013-304446>.
- Shao, L., Gao, Q., Xie, C., Fu, J., Xiang, M., & He, Y. (2020). Directly coaxial 3D bioprinting of large-scale vascularized tissue constructs. *Biofabrication*, 12, 1–12.
- Shapira, A., Kim, D. H., & Dvir, T. (2014). Advanced micro- and nanofabrication technologies for tissue engineering. *Biofabrication*, 6, 20301.
- Sodian, R., Fu, P., Lueders, C., Szymanski, D., Fritsche, C., Gutberlet, M., . . . Hetzer, R. (2005). Tissue engineering of vascular conduits: Fabrication of custom-made scaffolds using rapid prototyping techniques. *The Thoracic and Cardiovascular Surgeon*, 53, 144–149.
- Sodian, R., Loebe, M., Hein, A., Martin, D. P., Hoerstrup, S. P., Potapov, E. V., . . . Hetzer, R. (2002). Application of stereolithography for scaffold fabrication for tissue engineered heart valves. *ASAIO Journal (American Society for Artificial Internal Organs: 1992)*, 48, 12–16.
- Soliman, S., Sant, S., Nichol, J. W., Khabiry, M., Traversa, E., & Khademhosseini, A. (2011). Controlling the porosity of fibrous scaffolds by modulating the fiber diameter and packing density. *Journal of Biomedical Materials Research. Part A*, 96, 566–574.
- Song, S.-J., Choi, J., Park, Y.-D., Hong, S., Lee, J. J., Ahn, C. B., . . . Sun, K. (2011). Sodium alginate hydrogel-based bioprinting using a novel multinozzle bioprinting system. *Artificial Organs*, 35, 1132–1136.
- Stark, J. (1998). The use of valved conduits in pediatric cardiac surgery. *Pediatric Cardiology*, 19, 282–288.
- Subbiah, T., Bhat, G. S., Tock, R. W., Parameswaran, S., & Ramkumar, S. S. (2005). Electrospinning of nanofibers. *Journal of Applied Polymer Science*, 96, 557–569.
- Tan, Z., Wang, H., Gao, X., Liu, T., & Tan, Y. (2016). Composite vascular grafts with high cell infiltration by co-electrospinning. *Materials Science & Engineering. C, Materials for Biological Applications*, 67, 369–377.
- Tasoglu, S., & Demirci, U. (2013). Bioprinting for stem cell research. *Trends in Biotechnology*, 31, 10–19.
- Wang, J., Xie, H., Weng, Z., Senthil, T., & Wu, L. (2016). A novel approach to improve mechanical properties of parts fabricated by fused deposition modeling. *Materials & Design*, 105, 152–159.
- Wang, Y., Kibbe, M. R., & Ameer, G. A. (2013). Photo-crosslinked biodegradable elastomers for controlled nitric oxide delivery. *Biomaterials Science*, 1, 625–632.
- Wu, H., Fan, J., Chu, C. C., & Wu, J. (2010). Electrospinning of small diameter 3-D nanofibrous tubular scaffolds with controllable nanofiber orientations for vascular grafts. *Journal of Materials Science. Materials in Medicine*, 21, 3207–3215.
- Wu, P., Nakamura, N., Morita, H., Nam, K., Fujisato, T., Kimura, T., & Kishida, A. (2019). A hybrid small-diameter tube fabricated from decellularized aortic intima-media and electrospun fiber for artificial small-diameter blood vessel. *Journal of Biomedical Materials Research – Part A*, 107A, 1064–1070.
- Xu, C., Chai, W., Huang, Y., & Markwald, R. R. (2012). Scaffold-free inkjet printing of three-dimensional zigzag cellular tubes. *Biotechnology and Bioengineering*, 109, 3152–3160.

- Xu, T., Gregory, C. A., Molnar, P., Cui, X., Jalota, S., Bhaduri, S. B., & Boland, T. (2006). Viability and electrophysiology of neural cell structures generated by the inkjet printing method. *Biomaterials*, 27, 3580–3588.
- Xu, T., Jin, J., Gregory, C., Hickman, J. J., & Boland, T. (2005). Inkjet printing of viable mammalian cells. *Biomaterials*, 26, 93–99.
- Yamazoe, H., & Tanabe, T. (2009). Cell micropatterning on an albumin-based substrate using an inkjet printing technique. *Journal of Biomedical Materials Research. Part A*, 91, 1202–1209.
- Ye, L., Wu, X., Duan, H. Y., Geng, X., Chen, B., Gu, Y. Q., . . . Feng, Z. G. (2012). The in vitro and in vivo biocompatibility evaluation of heparin-poly(epsilon-caprolactone) conjugate for vascular tissue engineering scaffolds. *Journal of Biomedical Materials Research. Part A*, 100, 3251–3258.
- Zein, I., Hutmacher, D. W., Tan, K. C., & Teoh, S. H. (2002). Fused deposition modeling of novel scaffold architectures for tissue engineering applications. *Biomaterials*, 23, 1169–1185.
- Zhang, H., Jia, X., Han, F., Zhao, J., Zhao, Y., Fan, Y., & Yuan, X. (2013). Dual-delivery of VEGF and PDGF by double-layered electrospun membranes for blood vessel regeneration. *Biomaterials*, 34, 2202–2212.
- Zhang, Y., Yu, Y., Akkouch, A., Dababneh, A., Dolati, F., & Ozbolat, I. T. (2015). In vitro study of directly bioprinted perfusable vasculature conduits. *Biomaterials Science*, 3, 134–143.
- Ziabari, M., Mottaghtalab, V., & Haghi, A. (2010). A new approach for optimization of electrospun nanofiber formation process. *Korean Journal of Chemical Engineering*, 27, 340–354.

3D PRINTING AND PATTERNING VASCULATURE IN ENGINEERED TISSUES

10

Bagrat Grigoryan and Jordan S. Miller

Department of Bioengineering, Rice University, Houston, TX, United States

10.1 INTRODUCTION

Over the past half-century, researchers have made significant progress in the isolation and growth of human cells outside the body by deducing the characteristics of the extracellular environment (both soluble and insoluble) that contribute to the survival and growth of cells (Albrecht-Buehler, 1976). Moreover, the detailed mechanistic understanding of cellular biochemical activity has progressed at a rapid pace, powered by advanced genetic reporters (Shaner et al., 2005) and imaging modalities (Kanchanawong et al., 2010). However, efforts to adapt the findings from cell monolayer culture to the level of large tissues and organs have been hampered by technological limitations in keeping large masses of cells alive. While millions or perhaps even billions of human cells can now be grown and expanded as monolayers in Petri dishes, the field of bioengineering has no generic set of technologies for standardized assembly of cells into functional tissues or organ structures. The remaining major technological challenges are rooted in questions of tissue architecture and mass transport—how do we get nutrients and oxygen in and metabolic waste products out of tissues at rates akin to that seen in the human body (Miller, 2014; Hasan et al., 2014)? Here, we highlight recent efforts to fabricate vascular networks in engineered tissues to address questions of mass transport and blood perfusion. We also discuss some of the technical hurdles and conceptual targets on the horizon.

10.1.1 MACROPOROUS CONSTRUCTS AS TISSUE TEMPLATES

To enable convective transport within biocompatible materials, common approaches have utilized various material processing steps such as critical point drying (Dagalakakis et al., 1980), gas foaming and salt leaching (Jun and West, 2005), or electrospinning (Pham et al., 2006) to create macroporous structures that can be perfused *in vitro* for tissue culture. Indeed, these types of porous foams have shown great utility serving as templates for the construction of functional tissue extensions because they match the mechanical compliance of native tissue while also having high surface area in which nutrients and oxygen can be delivered (Yannas et al., 1982). However, precise spatial control of scaffold architecture is not easily achieved with these methodologies (Harley et al., 2010).

Soft lithography, a suite of techniques adapted from the microprocessor industry, offered spatial control of scaffold architecture through photopatterning with photolithography. With these technologies of patterning and then replica molding patterns into silicone substrates, detailed studies could be conducted at the cell-material interface (Whitesides et al., 2001; Kane et al., 1999). Early soft lithography and photolithography work involved fabricating substrates with grooves and microbeds surfaces, and chemical modifications to the substrate surface, to study cell adhesion, migration, and proliferation (Truskett and Watts, 2006). These approaches generally resulted in planar scaffolds to elucidate topological effects on cell behavior.

To control the cellular microenvironment in a more 3D fashion, Vozzi et al. developed macroporous scaffolds out of poly(dimethylsiloxane) (PDMS) templates which were cast into poly(L-lactic-co-glycolic acid) (PLGA) multilayer scaffolds (Figure 10.1) (Vozzi et al., 2003). Fabrication of macroporous scaffolds containing living cells was investigated by Liu et al. by combining tissue engineering with photolithographic methods (Liu and Bhatia, 2002). An apparatus was designed in which the prepolymer solution was injected into a chamber, with specified height controlled by spacer thickness, and then a mask was placed on top of the chamber and exposed to UV light (Figure 10.1). Multilayered hydrogel

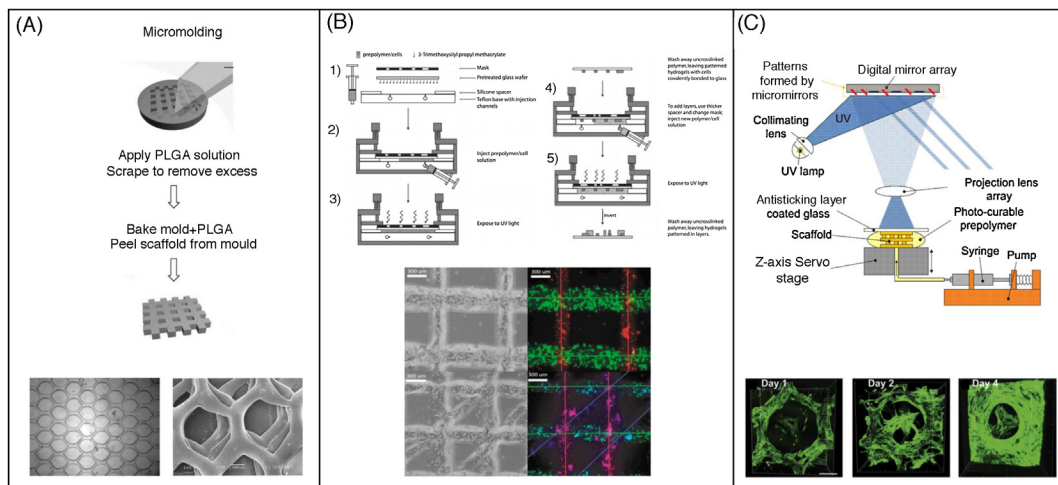


FIGURE 10.1

Patterned macroporous scaffolds can be fabricated by various methods. (A) Top: Micromachined masters can be replica-molded into multilayered microfluidic devices. Bottom: Single-layered and multilayered 3D PLGA scaffolds. Scale bars = 200 μm ; figure adapted from Vozzi et al. (2003). (B) Top: Methodology for additive photopatterning with physical photomasks to fabricate cell-laden hydrogels with macroscale features. Bottom: Fluorescent images of photopatterned cell-laden hydrogels with various macroscale patterns. Scale bar = 500 μm ; figure adapted from Liu Tsang et al. (2007). (C) Top: Dynamic photomasking with a computer-controlled digital mirror projection stereolithography system can be used to engineer multilayered structures in a highly automated fashion. Bottom: Cell infiltration of a 3D hexagonal printed scaffold over the course of 4 days. Scale bar = 100 μm ; figure adapted from Gauvin et al. (2012).

structures were formed after rinsing the first layer with buffer, changing the mask, using a thicker spacer, and injecting prepolymer solution into the chamber followed by UV exposure. Multilayered hydrogel microstructures containing encapsulated living cells were developed by adding cells to the prepolymer solution and following the same procedure. Using this approach, cellular tissue constructs were created in which placement of cells could be spatially controlled in 3D configurations throughout a thick construct. The authors were able to demonstrate fidelity of the patterns and cell viability in the fabricated hydrogel microstructures.

Further efforts from the same group resulted in a more refined apparatus to fabricate structures with macroporous architecture (Liu Tsang et al., 2007). Due to their interest in fabricating hepatic tissues, the authors tailored the chemistry and architecture of hydrogels to support hepatocyte survival and liver-specific function. The authors observed that coculture of hepatocytes with fibroblasts stabilized the hepatocytes, allowing for better incorporation into biomaterials by increasing their affinity to ligands. Multilayered cellular hydrogels fabricated with controlled 3D microarchitecture facilitated the transport of oxygen and nutrients, resulting in higher cell viability due to the high metabolic demands of encapsulated hepatocytes. Additionally, the authors demonstrated the importance of incorporating macroporous structures within the fabricated multilayered constructs after the constructs were transferred to a perfused bioreactor, resulting in higher cellular metabolic activity for 2 weeks compared to static constructs. Although the system utilized was able to achieve 3D microarchitectures that were reminiscent of native tissue, the approach involved manually switching out spacers, masks, and adding prepolymer solution which can be time consuming and increase the chances of problems with architectural fidelity. Similar 3D constructs were automatically fabricated in a layer-by-layer fashion by using 3D projection stereolithography to fabricate an entire layer under one single UV exposure (Gauvin et al., 2012). This approach resulted in rapid fabrication of tissue constructs with controlled microarchitecture. The custom designed projection stereolithography (PSL) system developed consists of a digital light processing (DLP) chip to create dynamic photomasks from computer-aided design (CAD), which are projected onto the prepolymer solution, and a servo stage which can increment the z-axis for patterning of subsequent layers (Figure 10.1). Additionally, a syringe which dispensed prepolymer solution was connected to the system. The authors demonstrated that the physical characteristics of the gelatin methacrylate (GelMA) scaffold, such as porosity and interconnectivity, can be tailored by controlling pore geometry and architecture. The authors also demonstrated that dynamic seeding of cells on the hydrogel structure remained viable, spread, and proliferated for an extended period of incubation, indicating biocompatibility of the fabrication process. Recently, similar liver-mimetic 3D structures were fabricated by 3D printing as a potential detoxification device. In this work, poly(diacetylene) nanoparticles were incorporated into hydrogels with precise microstructures and were shown to attract, capture, and sense toxins while the 3D matrix trapped the toxins (Gou et al., 2014).

10.1.2 FABRICATING FLUIDIC NETWORKS WITHIN BIOMATERIALS

The fabrication of macroporous scaffolds, such as those described earlier, provided early insights into the relationship between structure and function in native tissues, though they failed to mimic the cardiovascular system responsible for the bulk of convective mass transport in the body. Recent efforts have turned toward the introduction of vascular networks into engineered tissues, able to support larger cell populations, and able to sustain the flow of complex fluids such as whole blood.

Importantly, blood is known to be extremely sensitive to small perturbations in fluid dynamics and microenvironment, rapidly leading to clotting (McGuigan and Sefton, 2007). Here we discuss approaches to pattern perfusable channels and networks in engineered tissues, motivated by limitations in controlling individual pore size, shape, arrangement, and interconnectivity of microdevices.

Photolithography can typically pattern only a single layer of material at a time. Thus, strategies combining photolithographic processes with layer-by-layer lamination methods represent an important approach to achieve 3D scaffolds with high-resolution microstructures. To this end, Bettinger et al. developed a vasculature construct out of poly(glycerol-sebacate) (PGS), a tough, biodegradable elastomer with superior mechanical properties (Bettinger et al., 2005). Multiple layers of PGS vasculature construct were obtained by stacking and aligning layers, followed by a thermal bonding process. Although the authors demonstrated that cells seeded on the fabricated scaffold resulted in normal metabolic activity, the ability to encapsulate cells in these constructs was limited by the harsh fabrication process. Furthermore, the ability to precisely form microstructures with encapsulated cells in complex 3D structures cannot easily be obtained using this approach.

King et al. developed a novel technique for fabrication of high-resolution, high-precision features by combining a thermal fusion bonding process after fabrication of a single layer of microfluidic network composed of thermoplastic PLGA (King et al., 2004). This optimized approach involves conventional photolithography and silicon micromachining of single layers, followed by precise alignment and stacking of interconnected micropatterned films and thermal sealing, to obtain highly branched, multilayer PLGA microfluidic networks. Moreover, the authors were able to demonstrate the formation of 2 μm wide channels, close to the physical limits of traditional photolithography, all the way up to channels in the millimeter range. When perfused, single-layer networks showed no sign of leaks, occlusions, or channel-to-channel cross-talk, and demonstrated linear pressure-flow characteristics. Lactide has also been incorporated into a copolymer with PEG to form microchannels by selective degradation of bulk photopolymerized hydrogel substrates (Chiu et al., 2009). While the bulk of the scaffold was made from poly(ethylene glycol) diacrylate (PEGDA) by exposing light without a photomask, a photomask was used when PEG-PLLA-DA was injected into the system to create smaller patterns on top of the previous layer. Then, the spaces between the patterned strips were injected with PEGDA and cured. Channels were obtained in scaffolds by repeating the steps to create multilayered constructs and then incubating the construct in high pH environment to accelerate degradation of the PEG-PLLA-DA copolymer. This route for microchannel fabrication offers a 3D architecture with interconnected microchannels that can be obtained from biocompatible materials.

Inspired by the complex, multiscale microvascular network of leaf venation, He et al. fabricated nature-inspired microfluidic networks for perfusable tissue constructs (He et al., 2013). Their technique involved a microreplication method to mimic the microvascular network of natural leaf in synthetic substrates by digesting leaves to expose venation networks, sputtering with a thin layer of chrome, and then using the chrome-coated leaf venation as photomask to obtain a silicon mold with leaf venation to fabricate biomaterial hydrogels with multiscale vascular channels ranging from 30 μm to 1 mm in diameter. Seeding cells in this system resulted in high viability of endothelial cells, proliferation, and spreading along the microfluidic channels during the short culture period. The channels were perfused by mounting the hydrogels on an incline plate and introducing fluid from an inlet in a drop-wise manner, whose flow was induced by gravity. Further work by Wu et al. studied the transport efficiency of microvascular networks inspired by the hierarchical,

bifurcating network of leaf venation (Wu et al., 2010). In this approach, a CAD image of leaf venation was obtained and then a modified direct-write assembly process utilizing fugitive organic ink was used to create biomimetic microvascular networks of varying design. The authors explored the effects of network design on fluid transport efficiency and found that fluid transport efficiency is maximized when the network architecture obeys Murray's law, which relates the radii of branching vasculature to volumetric flow and velocity profiles showing maximal efficiency of mass transport (Murray, 1926; Sherman, 1981).

10.1.3 APPROACHES TO FABRICATE ENDOTHELIALIZED AND CELL-LADEN TISSUE CONSTRUCTS

Initial efforts to incorporate cells into constructs that resemble vasculature involved seeding of endothelial cells (ECs) inside channels after fabrication of 3D constructs. Lining channels with endothelial cells is critical for control of vascular functions such as barrier function, blood vessel formation, coagulation, and inflammatory response. To this end, Golden et al. have fabricated perfusable microfluidic extracellular matrix (ECM) hydrogels by using molded gelatin as a sacrificial element for the transport of materials in a tissue analogue (Golden and Tien, 2007). By encapsulating micromolded meshes of gelatin inside a gel followed by removal of gelatin by heating and flushing, interconnected channels as narrow as 6 μm were obtained. These gels were then seeded with microvascular endothelial cells (MECs) to demonstrate attachment, spreading, and proliferation of cells on the lumen of the channels, indicating normal function of seeded cells, which could later be perfused. To produce a multiplanar network that can be used to transport and exchange materials in a 3D manner, the authors coencapsulated and melted multiple gelatin meshes. Although soft lithography and photolithography techniques have been extensively used to create 3D microfluidic networks, one limitation of these techniques is that the fabricated channels contain a rectangular cross-section and sharp transitions between channels, which do not mimic physiological properties. Furthermore, the presence of rectangular walls and corners, in addition to nonphysiological blood flow conditions, results in asymmetric shear stress around the vessel perimeter, limiting the ability to form confluent endothelial cell layers. To this end, Borenstein et al. used an electroplating process to obtain sheets with semicircular cross-sections and smooth transitions at bifurcations and changes in diameter (Borenstein et al., 2010). To obtain closed circular microchannel networks, the sheets were aligned and bonded by applying an adhesive to the outer edges of the sheets. Cell viability and spreading of human umbilical vein endothelial cells (HUVECs) cultured within the circular channel networks was confirmed after 24 h of culture in channels containing multiple bifurcations of various diameters.

Although the techniques just discussed have been successful in incorporating ECs inside channel lumens, to mimic the more complex architecture of vasculature, various methods have been utilized to fabricate perfusable, cell-laden 3D constructs. One such method, developed by Cuchiara et al., involved a simple and robust multilayer replica molding technique in which PDMS and PEGDA are serially replica molded to develop microfluidic hydrogel networks embedded within independently fabricated PDMS (Cuchiara et al., 2010). Taking rational network design into account, the authors sought to determine the optimal microfluidic vessel spacing to maintain cell viability and maximize construct metabolic density of encapsulated cells to overcome diffusional limitations of nutrients and waste. Indeed, the viability of 3T3 fibroblasts was shown to depend on culture time, distance from perfused channel, and culture conditions. In constructs containing a perfused channel, necrotic cores were

shown to be confined to the outermost regions of the scaffold. Needle-based molding techniques have also been utilized to obtain perfusable channels and offer researchers a way to develop experimental models for studying vasculature function with a single channel. Chrobak et al. utilized this approach to generate open tubes of microvascular cells in a collagen gel with vessel diameters ranging from 75 to 150 μm (Chrobak et al., 2006). To obtain microvascular channels, a needle was suspended in a silicone mount while collagen was added into the chamber, surrounding the needle. After the collagen was allowed to gel, the needle was carefully removed to reveal a fluidic channel that matched the dimensions of the needle. By etching the tip of a needle, resulting in reduction of the needle diameter at the tip, abrupt changes in the diameter of vessel can be obtained. Using this technique, the authors demonstrated organization of seeded ECs in the tubes, which allowed the tubes to be perfused without significant leakage (Figure 10.2F). In addition to demonstrating the long-term culture of ECs for up to

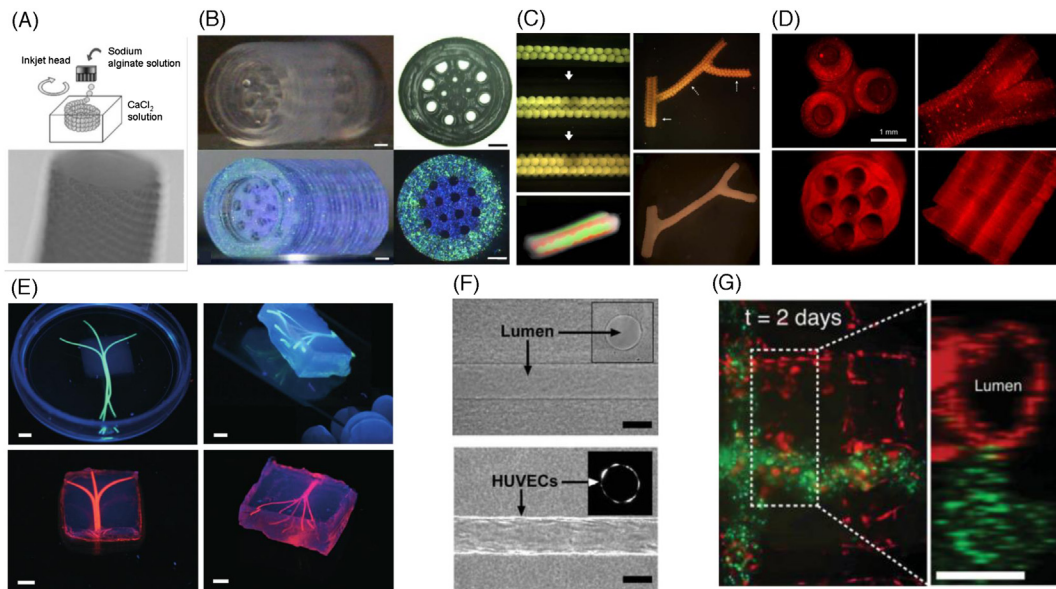


FIGURE 10.2

Microchannel structures with various architecture. (A) Fabrication of tubular structures composed of alginate hydrogel by ink-jet 3D bioprinting. Figure adapted from Nakamura et al. (2008). (B) Multilumen PEG hydrogel conduit obtained by line-scan stereolithography. Scale bars = 1 mm; figure adapted from Arcaute et al. (2006). (C) Assembly of multicellular spheroids into tubular structures with fusion of spheroids after a week in culture can be used to create branched architecture. Figure adapted from Norotte et al. (2009). (D) Fluorescent images of branched and multilumen hyaluronic acid hydrogel fabricated by projection stereolithography. Scale bar = 1 mm; figure adapted from Suri et al. (2011). (E) 3D-printed agarose fibers serve as temporary templates for casting perfusable vasculature. Scale bars = 3 mm; figure adapted from Bertassoni et al. (2014). (F) Endothelial cells can be lined along the lumen of a microchannel, which was fabricated by a needle-based casting approach. Scale bars = 100 μm ; figure adapted from Chrobak et al. (2006). (G) Fluorescent images of vascularized, heterogeneous tissue constructs fabricated by using a 3D bioprinter with multiple independently controlled printheads. Scale bar = 300 μm ; figure adapted from Kolesky et al. (2014).

3 weeks, the tubes exhibited normal microvascular functions such as adhesion of leukocytes and response to inflammatory mediators. Additionally, the authors demonstrated feasibility of incorporating cells into the gel by mixing cells with the collagen solution before gelation. Materials such as reconstituted collagen gels often exhibit weak mechanical strength and may not support physiologic flow rates through perfused vasculature. Synthetic materials provide an intriguing alternative, especially when functionalized with photoreactive groups. Photopolymerized hydrogels allow for straightforward tuning of mechanical properties by varying system parameters such as polymer concentration, light intensity, and photoinitiator concentration. To this end, Nichol et al. utilized the needle-based approach to demonstrate GelMA as a potential biomaterial for applications in microfluidic system and fabrication of vascularized engineered tissues (Nichol et al., 2010). Cell-laden constructs with endothelial-lined microchannels were obtained by UV radiation of a solution of GelMA within a rectangular PDMS mold containing a needle. After the needle was withdrawn, resultant gels were perfused and evidence of endothelial cell adhesion and aggregation within surrounding cells, in addition to elongation and reorganization of encapsulated 3T3 cells, was evident. Detailed studies quantifying the toxicity of photoinitiators and light sources have previously been performed (Bryant et al., 2000), allowing the experimentalist to retain cell viability during cell encapsulation.

10.1.4 APPROACHES TO INTEGRATE PATTERNED VASCULATURE *IN VIVO*

Although photolithographic and needle-based approaches to fabricate simple models for studying vasculature function have been successful, more advanced constructs are desirable which closely resemble the heterogeneity and complexity of tissues. Thus, techniques such as ink-jet printing and stereolithography have advanced significantly, providing researchers with high-resolution printing capability and an expanded parameter space of materials from which to choose. Early efforts in ink-jet printing involved fabricating tubular hydrogel structures with a liquid aqueous gelling medium (Nakamura et al., 2008). In this technique, droplets ejected from a print head containing sodium alginate form into alginate microgel beads by contact with calcium ions and fuse to form fibers and sheets as the ink-jet system is moved laterally and vertically (Figure 10.2A). Microgel beads as small as 10 μm in diameter were produced using this approach. To achieve 3D tubular structures on a very small scale, the ink-jet head was moved in a circular pattern as droplets of alginate were ejected onto the substrate in a layer-by-layer fashion to form long tubular structures. The group has also fabricated fibers, 2D sheets, multi-layered sheets, and more 3D vessels using this approach (Nishiyama et al., 2009). However, Cui et al. have utilized thermal ink-jet printing technology for microvascular fabrication and have demonstrated alignment and proliferation of cells inside a fibrin substrate (Cui and Boland, 2009). In this study, a fibrin scaffold was printed with cells, resulting in minimal deformation of the scaffold and little, if any, damage to cells. Fabricated microvasculature structures composed of fibrin and ECs resulted in the cells forming contacts with each other and, ultimately, aligning themselves along the fibrin channel to form a confluent lining. In addition, long-term scaffold integrity of the printed microvasculature was demonstrated.

Further techniques to fabricate even more complex heterogeneous structures layer-by-layer involve utilizing CAD-based rapid prototyping methods such as stereolithography. Chan et al. used a custom-modified stereolithography apparatus to fabricate complex 3D structures from photopolymerization of PEG by repetitive deposition and processing of individual layers (Chan et al., 2010). In addition, the authors characterized the penetration depth and critical exposure energy parameters

for ideal sequential layer-by-layer hydrogel photopatterning so that layers sufficiently adhere to each other and reduce overcuring of printed layers, as well as swelling behavior and mechanical properties of the laser polymerized PEGDA hydrogels. The authors were also able to demonstrate control over spatial distribution of cells in a multilayered structure with minimal mixing as well as high viability, proliferation, and spreading of entrapped cells, indicating that this methodology can be applied to achieve high spatial control to recapitulate complex 3D tissue microarchitecture. Tissue constructs with open channels can also be obtained by using a light-based projection stereolithography system due to the high fabrication rate and resolution of this technique in printing layer-by-layer (Lin et al., 2013). However, solutions containing cells in the apparatus chamber tend to settle down over time during the printing process due to the inherent higher density of the cells, resulting in constructs with nonhomogenous encapsulation of cells. To address this issue, the authors included optimized concentration of Percoll to increase the density of the precursor solution to match the density of the cells, making them neutrally buoyant. However, addition of Percoll resulted in some undesirable curing since the authors observed that fabrication of porous structures resulted in partial pore occlusion. The authors also fabricated open channel scaffolds with encapsulated cells and observed high cell viability and metabolic activity in the fabricated hydrogels, even though the scaffolds did not contain any adhesive peptides.

Other methods for fabricating vessel-like tissue constructs involve dispensing cell containing macrofilaments or spheroids and then allowing the cells to fuse into whole tissue constructs. One strategy for direct-write bioprinting of a cell-laden ECM hydrogel dispensed prepolymerized cell laden GelMA hydrogel fibers from glass capillary tubes (Bertassoni, Cardoso, et al., 2014). The bioprinting process involves aspirating the hydrogel precursor into a glass capillary by the upward movement of a metallic piston, followed by photopolymerization of the precursor inside the capillary by exposure to UV light, and then dispensing the hydrogel fiber as the metallic piston is pushed down against the cross-linked gel. The authors demonstrated bioprinting of macroscale 3D cell-laden constructs by positioning hydrogel fibers in one plane and stacking a second layer of perpendicular fibers on the plane above. More complex constructs were fabricated that contained GelMA hydrogel blocks with impregnated planar and 3D bifurcating fiber networks to achieve tissue analogs that mimic vasculature. To fabricate constructs with hollow fibers, a sacrificial layer was dispensed during the printing process and layer removed, resulting in microchannels within the fabricated construct. The authors demonstrated relatively high cell viability and proliferation rates of encapsulated cells in the printed gels.

Additionally, other direct-write processes utilize a scaffold-free approach to circumvent some hurdles in tissue engineering approaches that utilize a biomaterial scaffold, such as a plethora of materials to choose from, constructing viable tissues with high cell density, and potential uncontrollable scaffold mechanical effects on cell behavior. Thus, utilization of a scaffold-free approach can avoid some of these issues. Furthermore, a scaffold-free approach is inspired by the self-assembly process of cells during early morphogenetic processes, in which individual cells organize into multicellular subunits (Chang et al., 2013). To fabricate bioartificial vessel-like grafts using a scaffold-free approach, an open-source printer, modified to hold microcapillary tubes for bioprinting of hydrogel macrofilaments, was used to dispense cylindrical macrofilaments layer-by-layer (Skardal et al., 2010). Culture of the filaments resulted in discrete structures, as the filaments fused with each other to form a continuous structure. With this approach, the resolution of the printed tissue construct was determined by the capillary internal diameter, which was 500 μm . To better facilitate the fusion of dispensed cell-containing materials, Jakab et al. utilized a scaffold-free approach by dispensing multicellular spheroids and cylinders to allow cells to self-organize into functional living structures of prescribed shape (Jakab et al., 2008). Multicellular spheroids and cylinders were prepared as bioink and

deposited with print heads onto biopaper containing a collagen gel, which serves as building blocks of a molding template. The approach involves centrifuging trypsinized cells and then incubating them in capillary micropipettes, which are transferred into an apparatus that extrudes a cell-laden fiber that is automatically cut into equal-sized cylinders. After culturing for 60 h, the authors observed fusion of cylindrical aggregates into well-defined tubular structures. More advanced constructs, such as branched tubes that better resemble vessels, were also obtained using this method. Further work using cylindrical bioink to obtain multilayered tubular vascular grafts has been developed by the same group, demonstrating versatility of the approach (Norotte et al., 2009). Postprinting fusion of the cellular cylinders was observed to take place in 2–4 days, as opposed to the 5–7 days for spheroids, and resulted in formation of uniform tubes (Figure 10.2C). However, the authors observed sparsely distributed apoptotic cells throughout the vascular wall of their engineered vessels.

On-demand capability of 3D bioprinting techniques to fabricate large, clinically relevant 3D structures at high resolution could permit patient-specific, highly customized tissue constructs. Initial work on fabricating larger multilayered tissue conduits with multiple lumens utilizing stereolithography techniques was achieved by Arcaute et al. (Arcaute et al., 2006) (Figure 10.2B). Large blocks comprising 5 cm in length with embedded channels with a bifurcation were fabricated to demonstrate the feasibility of obtaining geometries representative of the arterial system. The authors demonstrated high cell viability in encapsulated hydrogels photocrosslinked using SL over 24 h, indicating that the cells can survive the stereolithography process. Progress to obtain branched constructs was demonstrated by Suri et al. who used stereolithography to fabricate 3D scaffolds of hyaluronic acid with defined architecture mimicking the native tissue microenvironment (Suri et al., 2011). This approach involves a digital-mirror device (DMD) to create structures in a layer-wise fashion by fabricating an entire layer simultaneously. Hyaluronic acid scaffolds with different geometries with varying pore shapes and sizes were fabricated in a layer-by-layer fashion and were shown to retain functionality after the fabrication process (Figure 10.2E). The authors also demonstrated that the fabricated scaffolds support cell adhesion, spreading, and proliferation after seeding the branched scaffolds. While the scaffolds were designed for nerve tissue engineering purposes, similar clinically relevant large-scale scaffolds can be fabricated for vascular applications. Duan et al. implemented an onboard photocrosslinking system on an extrusion-based open-source printer to enable simultaneous 3D extrusion printing and curing of PEG hydrogels with a mounted UV-LED cross-linking module (Duan et al., 2014). By doing so, a solution was gelled immediately after dispensing and onset of light exposure, without requiring a biopaper to initiate gelation. Trileaflet valve hydrogels were fabricated to test the response of encapsulated human valvular interstitial cells (VICs) within the printed leaflets. The authors observed that encapsulated cells remained spherical in shape after 3 days in culture but began to exhibit more spread morphology on day 7. The same system was used by the group to rapidly engineer complex, heterogeneous aortic valve scaffolds (Hockaday et al., 2012). The authors printed an axisymmetric aortic valve geometry, including the sinus and leaflets, to replicate native valve geometry and regional mechanical heterogeneity. They characterized the mechanical properties of hydrogels and observed high viability of cells seeded onto the valve scaffold for over 3 weeks. The authors also demonstrated the ability to print a complex model obtained from a medical image, suggesting the use of this technology for printing of patient-specific tissue constructs. Additionally, the same group demonstrated the versatility of the approach by using another material system to fabricate mechanically robust living trileaflet valves with multiple cell populations using 3D printing (Duan et al., 2013). In simple alginate/gelatin hydrogels, high cell viability for encapsulated VICs and smooth muscle cells (SMCs) was observed over 7 days in culture with specific markers for VIC expression. The authors fabricated a heterogeneous aortic valve

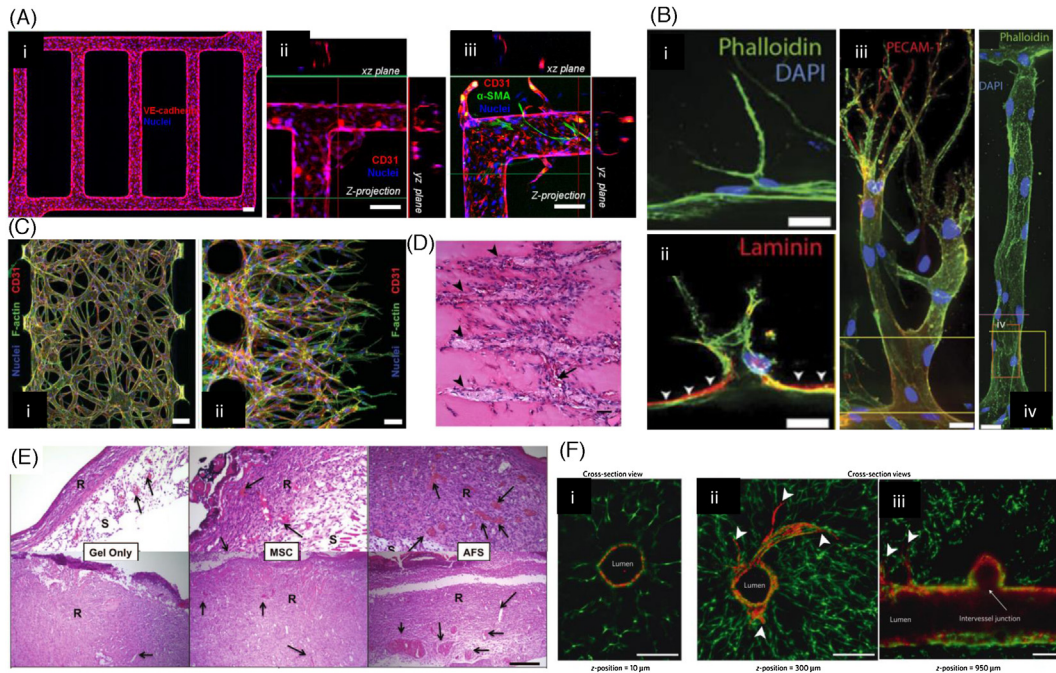
conduit with a root consisting of SMCs, which was deposited first, and then a leaflet region consisting of VICs, to demonstrate fabrication of living valve conduits with anatomical resemblance to the native valve based on alginate/gelatin hydrogel system via 3D bioprinting, obtaining 3D hydrogel constructs that can maintain high cell viability with clinically relevant thickness. This approach allowed the researchers to obtain anatomically complex, mechanically heterogeneous valve scaffolds rapidly, without any processing post-fabrication.

10.1.5 PATTERNING MULTISCALE VASCULATURE WITH ENDOTHELIAL FUNCTION

Although fluid flow through perfusable microvasculature can help mimic physiologic transport on a small scale, the mammalian cardiovascular system is comprised of a wide range of diameters of blood vessels connected through a well-organized fractal hierarchy. Researchers are also investigating ways to generate multiscale vasculature—vessel networks containing an array of channel diameters and their accompanying fluidic junctions.

Work utilizing a direct-writing approach involved fabricating microchannels with barrier function by casting a hydrogel precursor over bioprinted agarose fibers, which serve as template material (Bertassoni, Cecconi, et al. 2014). After photopolymerization of constructs, the template fibers can be removed to result in perfusable networks (Figure 10.2E). The authors demonstrated the ability to fabricate microchannel networks with various architectural features inside a range of photopolymerizable hydrogel systems. Microchannels with diameters ranging from 1000 μm down to 150 μm could be obtained using this approach. By parallel overlapping of multiple template fibers over one another, the authors demonstrated versatility of the approach to fabricate even larger channel diameters that branched out into lateral individual channels of narrower diameters. The authors also demonstrated high efficiency of bioprinted microchannels to form cell-laden tissue constructs with improved functionality due to the channels allowing for improved nutrient transport, resulting in increased cell viability and cell differentiation. Furthermore, formation of endothelial monolayers inside hydrogel constructs was observed, allowing the constructs to remain fully perfusable.

We have also demonstrated an approach to rapidly cast patterned vascular networks in engineered tissue, a technique compatible with a wide range of cell types, synthetic and natural ECMs, and cross-linking strategies (Miller et al., 2012). In this approach, rigid 3D filament networks of carbohydrate glass are printed, cast into an ECM, and then dissolved to obtain a monolithic cellularized tissue construct. Multiscale filament architecture was obtained by varying only the translational velocity of the extrusion nozzle. After sacrificing the carbohydrate glass, smooth elliptical intervessel junctions were left behind by the glass interfilament fusions. As a result, fluidic connections between adjoining vascular channels can be obtained. Encapsulation of cells in the ECM was achieved by mixing a suspension of cells in the ECM prepolymer, while endothelialization was achieved by injecting a suspension of ECs into the vascular architecture. Encapsulated cells were shown to be viable, spread, and migrate normally in the channeled scaffolds. Seeded ECs quickly lined the walls of the network, even in constructs containing vessels of differing diameters (Figure 10.3F). Complete endothelialization of the vascular scaffolds resulted in the ability to perfuse human blood under high pressure by either laminar or pulsatile flow. Additionally, we found that highly sensitive cells, such as primary hepatocytes, can maintain metabolic activity at or near physiologic cell densities in tissue constructs with perfusable vascular channels and junctions.

**FIGURE 10.3**

Demonstrating vasculogenic and angiogenic vessel formation in different systems. (A) Microfluidic vessel network formed by injection molding techniques, which was then stacked and sealed to result in an enclosed fluidic structure: (i) overall network structure, (ii) angiogenesis formation of sprouting endothelial cells, (iii) endothelial sprouting with perivascular interaction. Scale bars = 100 μm ; figure adapted from [Zheng et al. \(2012\)](#). (B) Endothelial cells shown to sprout over time (early sprouts: (i) and (ii); late sprouts: (iii)), forming into matured neovessels that are lumenized end-to-end (iv). Scale bars = 25 μm ; figure adapted from [Nguyen et al. \(2013\)](#). (C) Microfluidic device fabricated from soft lithography and replica molding: (i) cells encapsulated inside a channel demonstrate microvascular networks formed through vasculogenesis after 2 days in culture, (ii) cells coated inside a channel demonstrate angiogenic sprouts after 2 days in culture. Scale bars = 20 μm ; figure adapted from [Kim et al. \(2013\)](#). (D) Cords constructed from a microtissue molding approach demonstrating formation of new capillaries along the length of the patterned cords after 7 days of implantation (arrows point to sprouts, arrowheads point to cords). Scale bar = 25 μm ; figure adapted from [Baranski et al. \(2013\)](#). (E) Histological analysis of bioprinted cells onto skin wounds of a mice indicating thicker regeneration of tissue with more blood vessels in mesenchymal stem cell and amniotic fluid-derived stem cell treated patches compared to gel only (arrows point to vessels formed *in vivo*). Scale bars = 50 μm ; figure adapted from [Skardal et al. \(2012\)](#). (F) Perfusable 3D engineered tissues fabricated from casting of patterned networks: (i) Endothelial lining of microchannel surrounded by 10T1/2 cells after 9 days in culture, (ii) and (iii) endothelial sprouts from patterned vasculature at different positions along the same vessel network (arrowheads point to sprouts). Scale bars = 200 μm ; figure adapted from [Miller et al. \(2012\)](#).

10.1.6 ANGIOGENESIS, VASCULOGENESIS, AND *IN VIVO* INTEGRATION

Over the past few years, significant progress has been made to achieve perfusable microchannels containing cells; however, for translational applications, angiogenesis, vasculogenesis, and *in vivo* integration of

fabricated scaffolds will need to be demonstrated. Work by Zheng et al. involved the use of lithographic processes to form defined 3D constructs with endothelialized vessels with an emphasis on their biofunctionality *in vitro* (Zheng et al., 2012). Their methodology entailed creation of a microstructured silicone stamp, which served as a master, to mold collagen, injection and gelation *in situ* of collagen, followed by sealing of collagen layers to form an enclosed fluidic structure. Microfluidic vascular networks seeded with ECs demonstrated expression of endothelial phenotype and localization of cells to maintain cellular junctions between ECs, resulting in a microfluidic vascular network that exhibits a barrier function (Figure 10.3A). Interestingly, the cross-sectional area of the engineered microvessels changed profile from the original square cross-section defined lithographically toward an elliptical cross-section during the first 3 days of culture. Long endothelial sprouting containing lumens was evident in the ECM scaffold by 2 weeks in culture, indicating that the proposed system allows for the initiation of angiogenesis from native-like vessels. When microfluidic vascular networks were cocultured with ECs and perivascular cells, and subsequently perfused with vasculogenic media, the response of the networks to proangiogenic signals was found to be modulated by pericytes. Additionally, the authors demonstrated that perfusion of microfluidic vascular network with blood resulted in majority of platelets flowing past the endothelial surface without adhering. Kim et al. further studied the formation of vasculogenesis- and angiogenesis-derived microvascular networks by fabricating microfluidic devices using soft lithography and replica molding (Kim et al., 2013). The microfluidic chip design involved channels containing cells, partitioned by microposts, flanked with fluidic channels to emulate complex endothelial dynamics to grow native 3D vascular networks that can be readily perfused (Figure 10.3C). The approach yielded the presence of a continuous, hollow lumen along the length of the vessels with continuous cell–cell junctions lining the intersection of the endothelial cells. Quickly after seeding the microvascular networks with endothelial cells, the authors observed that the cells displayed elongated morphology and assembly into tubule-like structures encompassing a nascent lumen. Soon after, perfusable microvascular networks were obtained, followed by the enlargement of the lumen. Additionally, the authors were able to induce angiogenesis sprouting in the matrix as evidenced by tip cell formation and angiogenic sprouting along the openings of channels within 24 h of coculture with ECs and fibroblasts. After 3 days in culture, the leading tips were observed to have formed into lumenized vessels, establishing fluidic connections with flanking channels. To study potential flow-induced endothelial responses, the authors perfused the microvascular networks and observed that the fluid flow induced cytoskeletal reorganization of the ECs within a few hours and that networks perfused for only 1 h resulted in a significant increase in the synthesis of nitric oxide.

To further mimic angiogenesis processes in engineered tissue, Chiu et al. developed a method to engineer vascular network consisting of branching vessels that form a microvascular bed suitable for rapid vascularization of engineered tissues *in vitro* (Chiu et al., 2012). In this approach, biomaterials were used to create a niche that allowed sprouting and anastomosis of the vasculature from the branching vessels *in vitro* by control release of angiogenic and cardioprotective peptide T β 4. Micropatterned PDMS substrates were fabricated by standard soft lithography to obtain substrates consisting of lanes with varied width and height. A solution of hydrogel was then evenly distributed onto each substrate to coat the surface and allowed to gel. The authors exposed mouse arterial and venous explants to T β 4 encapsulated collagen-chitosan hydrogels and were interested in guiding and controlling the sprouting from explants through the integrated use of topographical cues involving different width grooves. Indeed, the authors observed that the enabling factor in tube formation and guidance was the presence of topographical cues, which was essential for the formation of branches with open lumens. The grooves not only provide physical guidance for the

sprouting capillaries, but also influence the local concentration of autocrine growth factors released from the ECs actively participating in angiogenesis. Organotypic models were also fabricated to investigate angiogenic sprouting and neovessel formation within a 3D ECM by flowing combinations of factors next to an endothelium-lined channel (Nguyen et al., 2013). In this approach, a 3D microfluidic device with two parallel channels was fabricated by casting collagen into a PDMS mold with needles placed across the casting chamber, allowing the collagen to polymerize, and then removing the needles to result in hollow cylindrical channels in the matrix. After ECs were seeded into one of the channels, the second channel was perfused with angiogenic factors to establish a gradient across the collagen matrix to the endothelium. The authors observed that when a complex combination of proangiogenic factors was perfused into the second channel, more substantial multicellular sprout-like structures were formed which mirror major steps of *in vivo* angiogenesis (Figure 10.3B). After 1 week of culture, tip cells breached the source channel and resulted in a connection between the two parallel channels. Perfusion studies showed that beads flowed through the neovessels without leakage into the interstitial space, indicating fully developed, continuously endothelialized lumens. Additionally, the authors observed maturation of secondary branches with their own new tip cells in the fabricated organotypic model.

In addition to observing angiogenesis and vasculogenesis *in vitro*, some efforts demonstrating these processes *in vivo* have emerged. One such technique, laser-induced forward transfer, involves transfer of material from a donor slide, covered with a laser-absorbing gold layer, onto a collector slide by pulsing the laser. Transfer of material to the collector slide is achieved through generation of high gas pressure, which propels material on the donor slide toward the lower collector slide. This technique was used by Gaebel et al. to create specific vascular patterns consisting of different cell types for cardiac regeneration (Gaebel et al., 2011). In addition to the gold layer, the donor slide also contained a layer of cell-containing material to be transferred while the collector slide contained a polyester urethane urea patch immersed in Matrigel®. A cardiac patch composed of polyester urethane urea was spatially patterned with ECs and mesenchymal stem cells (MSCs) *in vitro* and then transplanted to infarcted zone of rat hearts. After 8 weeks of implantation, the authors observed increased vessel formation and functional improvement of infarcted hearts of transplanted implants. Additionally, the authors mentioned that histological analysis revealed integration of the implanted patch with the host myocardium. Tissue constructs fabricated by ink-jet printing technology were also implanted into mice and then analyzed for vasculature formation by using MRI (Xu et al., 2008). In this approach, the print head consisted of calcium chloride and cells, while the chamber consisted of sodium alginate and collagen solution mixture. Upon ejection of the cell-calcium chloride drops, gelation occurred rapidly, resulting in construction of 3D structures. After implanting the printed tissue constructs, the authors used MRI with contrast perfusion to image the vasculature formation *in vivo* and performed histological analysis to determine host cell invasion into the transplanted construct. The authors observed a vascular network in the superficial area of the constructs printed with cells, which express EC-specific marker vWF, while few vascular networks were found in the cell-free implant.

Interestingly, Skardal et al. (2012) also printed patches directly onto the wound site to accelerate the healing process of large skin wounds. In this study, bioprinting technology was used to deposit fibrin/collagen gels, with either MSCs or amniotic-fluid-derived stem (AFS) cells, directly onto the defect site of mice. The printed patch provided full coverage over the wound area and evidence of re-epithelialization was observed by histological analysis (Figure 10.3E). Compared to a gel-only treatment, treatment with MSC and AFS resulted in thicker regenerating tissue with a greater number of

blood vessels observed in the regenerating skin. The AFS treatment resulted in larger, mature vessels that strongly stained for SMA and was found to contain RBCs inside the vasculature in the regenerating skin. However, the authors noted that since the cells in the printed patch remained at the wound surface, they did not migrate into the underlying tissue, thus indicating that the printed cells did not permanently integrate with the host tissue. In addition to patches, geometrically defined endothelial cords were developed and transplanted into mice by Baranski et al. (Baranski et al., 2013). Endothelialized cords were micropatterned using a PDMS mold and then transplanted to mice after the cords were polymerized between fibrin layers, encasing the cords. Indeed, formation of blood vessels, which were large and poorly organized on day 3 but remodeled into smaller, lumenized capillaries as early as 7 days, was evident in implanted endothelialized cords (Figure 10.3D). Additionally, the authors demonstrated that the cellular organization of the cords was vital for the vascularization response of the implant due to the presence of blood and vessels throughout the length of the cords. Anastomosis of neovessels was verified by perfusion studies and imaging of colabeled capillaries, which indicated the presence of chimeric composition of host and implanted cells in some vessels. The authors also developed endothelialized cords containing hepatocyte aggregates to demonstrate that the encapsulated cells exhibited enhanced hepatic function due to enhanced vascular supply as a result of neovessels formation along the tissue constructs.

10.1.7 ADVANCED TECHNOLOGIES WHICH MAY ASSIST IN VASCULAR TISSUE FABRICATION

Although much has been accomplished to fabricate 3D constructs with multiscale vasculature, 3D bioprinting technologies will need to advance to become more rapid, and achieve high throughput and resolution for translational purposes. Additionally, further techniques may need to be developed to pattern complex biochemical and biomechanical patterns within scaffolds to better mimic the *in vivo* environment.

To significantly increase the throughput in 3D bioprinting technology, Hansen et al. developed a method involving microvascular multinozzle print heads for printing of planar and multilayered architectures composed of single and multicomponent materials (Hansen et al., 2013). The multinozzle print heads are obtained by patterning a bifurcating network into a clear acrylic substrate using CNC machining, solvent-welding the patterned substrate to a flat acrylic substrate, resulting in a monolithic block that contains the embedded microvascular network. The authors were able to fabricate nozzle heads consisting of six branching generations, where each channel width and depth is 200 μm . The authors also demonstrated that their design is scalable for large-area, rapid fabrication of planar and 3D functional microarchitectures with microscale features. They also validated uniform ink-flow within the fabricated microvascular print heads to achieve high-fidelity, reproducible printed features. Further work for creating rapid vascularized, heterogeneous tissue constructs was also demonstrated by the same group by developing a custom-designed, large-area 3D bioprinter with four independently controlled print heads (Kolesky et al., 2014). These print heads contain inks consisting of cells and an aqueous fugitive ink that was removed from the final tissue construct. The authors showed the ability to print multiple cell types in the fabricated constructs, which are subsequently embedded in a pure GelMA matrix. 1D microchannel arrays were printed with final diameters ranging from 100 μm to 1 mm. Engineered construct channels were seeded with

endothelial cells, and after 2 days, high viability of cells as well as assembly into a nearly confluent layer were observed (Figure 10.2G).

In addition to obtaining tissue constructs rapidly and in a high-throughput manner, higher resolution 3D bioprinting systems will be desirable to obtain higher structural resolution, permitting printing of capillaries into constructs. By using two-photon laser scanning (TPLS), Ovsianikov et al. demonstrated the feasibility of obtaining 100 nm structural resolution (Ovsianikov et al., 2008). This technique is demonstrated to precisely reproduce scaffold structure and properties by using CAD-designed 3D scaffolds. Although the authors fabricated a 3D vascular microcapillary structure using this technique, their cellular studies were performed on much simpler structures made out of photosensitive organic–inorganic hybrid polymer ORMOCER and epoxy-based SU-8 materials. The authors showed that cells seeded on a cylindrical structure made out of these materials support cell growth and are biocompatible. Additionally, two-photon photopolymerization can be utilized for biochemical and biomechanical patterning of hydrogels to guide cell behavior (Hahn et al., 2006). Similar techniques were also utilized to fabricate 3D micropatterning of biomolecules immobilized in a preformed network for 3D spatial control of cell migration (Lee et al., 2008). To obtain 3D patterns of covalently immobilized biomolecules within hydrogel networks, cells encapsulated in fibrin clusters were photopolymerized into collagenase-sensitive PEG hydrogels. The hydrogels were then soaked in PEG-RGDS, and, finally, TPLS was used to irradiate hydrogels according to predesigned virtual patterns. By doing so, PEG-RGDS was conjugated in a 3D network of hydrogels in predetermined patterns, allowing the researchers to achieve microscale control of 3D arrangements of biomolecules and cells. Using this method, channels of RGDS were conjugated adjacent to fibroblast clusters to allow guided cell migration out from the cell clusters. Branching of cells and subsequent migration into two separate paths were observed when the migrating cells encountered an intersection of the patterned areas.

Innovations in patterning and 3D printing technologies have advanced the field of tissue engineering to fabricate engineered tissue constructs with complex microstructures. However, debates remain as to the extent of patterning that will be required for reconstructing whole tissue and organ replacements for human patients. Indeed, there are still many technological hurdles involving more precise control of architecture and mass transport for fabrication of clinically relevant tissue constructs. Hence, efforts must be focused on statistically significant explorations of tissue architecture which can be validated to direct future work.

References

- Albrecht-Buehler, G. (1976). Filopodia of spreading 3T3 cells. Do they have a substrate-exploring function? *The Journal of cell biology*, 69(2), 275–286. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2109684&tool=pmcentrez&rendertype=abstract> [Accessed July 1, 2014].
- Arcaute, K., Mann, B. K., & Wicker, R. B. (2006). Stereolithography of three-dimensional bioactive poly(ethylene glycol) constructs with encapsulated cells. *Annals of biomedical engineering*, 34(9), 1429–1441. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16897421> [Accessed February 28, 2014].
- Baranski, J. D., et al. (2013). Geometric control of vascular networks to enhance engineered tissue integration and function. *Proceedings of the National Academy of Sciences of the United States of America*, 110(19), 7586–7591. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3651499&tool=pmcentrez&rendertype=abstract> [Accessed June 20, 2014].

- Bertassoni, L. E., Cardoso, J. C., et al. (2014a). Direct-write bioprinting of cell-laden methacrylated gelatin hydrogels. *Biofabrication*, 6(2), 024105. Available at: <http://iopscience.iop.org/1758-5090/6/2/024105> [Accessed June 9, 2014].
- Bertassoni, L. E., Ceconi, M., et al. (2014b). Hydrogel bioprinted microchannel networks for vascularization of tissue engineering constructs. *Lab on a chip*, 20–22. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24860845> [Accessed May 27, 2014].
- Bettinger, C. J., et al. (2005). Three-Dimensional Microfluidic Tissue-Engineering Scaffolds Using a Flexible Biodegradable Polymer. *Advanced materials (Deerfield Beach, Fla.)*, 18(2), 165–169. Available at: <http://onlinelibrary.wiley.com/doi/10.1002/adma.200500438/full> [Accessed July 1, 2014].
- Borenstein, J. T., et al. (2010). Functional endothelialized microvascular networks with circular cross-sections in a tissue culture substrate. *Biomedical microdevices*, 12(1), 71–79. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19787455> [Accessed February 8, 2014].
- Bryant, S. J., Nuttelman, C. R., & Anseth, K. S. (2000). Cytocompatibility of UV and visible light photoinitiating systems on cultured NIH/3T3 fibroblasts in vitro. *Journal of biomaterials science. Polymer edition*, 11(5), 439–457. Available at: <http://www.tandfonline.com/doi/abs/10.1163/156856200743805> [Accessed July 1, 2014].
- Chan, V., et al. (2010). Three-dimensional photopatterning of hydrogels using stereolithography for long-term cell encapsulation. *Lab on a chip*, 10(16), 2062–2070. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20603661> [Accessed February 28, 2014].
- Chang, D. R., et al. (2013). Lung epithelial branching program antagonizes alveolar differentiation. *Proceedings of the National Academy of Sciences of the United States of America*, 110(45), 18042–18051. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3831485&tool=pmcentrez&rendertype=abstract> [Accessed May 29, 2014].
- Chiu, L. L. Y., et al. (2012). Perfusable branching microvessel bed for vascularization of engineered tissues. *Proceedings of the National Academy of Sciences of the United States of America*, 109(50), E3414–E3423. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3528595&tool=pmcentrez&rendertype=abstract> [Accessed January 28, 2014].
- Chiu, Y.-C., et al. (2009). Formation of Microchannels in Poly(ethylene glycol) Hydrogels by Selective Degradation of Patterned Microstructures. *Chemistry of Materials*, 21(8), 1677–1682. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2810413&tool=pmcentrez&rendertype=abstract> [Accessed June 9, 2014].
- Chrobak, K. M., Potter, D. R., & Tien, J. (2006). Formation of perfused, functional microvascular tubes in vitro. *Microvascular research*, 71(3), 185–196. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16600313> [Accessed February 28, 2014].
- Cuchiara, M. P., et al. (2010). Multilayer microfluidic PEGDA hydrogels. *Biomaterials*, 31(21), 5491–5497. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20447685> [Accessed February 24, 2014].
- Cui, X., & Boland, T. (2009). Human microvasculature fabrication using thermal inkjet printing technology. *Biomaterials*, 30(31), 6221–6227. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19695697> [Accessed January 20, 2014].
- Dagalakis, N., et al. (1980). Design of an artificial skin. Part III. Control of pore structure. *Journal of biomedical materials research*, 14(4), 511–528. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/7400201> [Accessed July 1, 2014].
- Duan, B., et al. (2013). 3D bioprinting of heterogeneous aortic valve conduits with alginate/gelatin hydrogels. *Journal of biomedical materials research. Part A*, 101(5), 1255–1264. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3694360&tool=pmcentrez&rendertype=abstract> [Accessed January 30, 2014].
- Duan, B., et al. (2014). Three-dimensional printed trileaflet valve conduits using biological hydrogels and human valve interstitial cells. *Acta biomaterialia*, 10(5), 1836–1846. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24334142> [Accessed June 16, 2014].

- Gaebel, R., et al. (2011). Patterning human stem cells and endothelial cells with laser printing for cardiac regeneration. *Biomaterials*, 32(35), 9218–9230. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21911255> [Accessed May 14, 2014].
- Gauvin, R., et al. (2012). Microfabrication of complex porous tissue engineering scaffolds using 3D projection stereolithography. *Biomaterials*, 33(15), 3824–3834. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3766354&tool=pmcentrez&rendertype=abstract> [Accessed January 22, 2014].
- Golden, A. P., & Tien, J. (2007). Fabrication of microfluidic hydrogels using molded gelatin as a sacrificial element. *Lab on a chip*, 7(6), 720–725. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17538713> [Accessed January 26, 2014].
- Gou, M., et al. (2014). Bio-inspired detoxification using 3D-printed hydrogel nanocomposites. *Nature communications*, 5, 3774. Available at: <http://www.nature.com/ncomms/2014/140508/ncomms4774/full/ncomms4774.html?message-global=remove> [Accessed May 26, 2014].
- Hahn, M. S., Miller, J. S., & West, J. L. (2006). Three-Dimensional Biochemical and Biomechanical Patterning of Hydrogels for Guiding Cell Behavior. *Advanced Materials*, 18(20), 2679–2684. Available at: <http://dx.doi.org/10.1002/adma.200600647>.
- Hansen, C. J., et al. (2013). High-throughput printing via microvascular multinozzle arrays. *Advanced materials (Deerfield Beach, Fla.)*, 25(1), 96–102. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23109104> [Accessed February 21, 2014].
- Harley, B. a, et al. (2010). Design of a multiphase osteochondral scaffold III: Fabrication of layered scaffolds with continuous interfaces. *Journal of biomedical materials research. Part A*, 92(3), 1078–1093. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19301263> [Accessed July 2, 2014].
- Hasan, A., et al. (2014). Microfluidic techniques for development of 3D vascularized tissue. *Biomaterials*, 35(26), 7308–7325. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S014296121400489X> [Accessed July 1, 2014].
- He, J., et al. (2013). Fabrication of nature-inspired microfluidic network for perfusable tissue constructs. *Advanced healthcare materials*, 2(8), 1108–1113. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23554383> [Accessed June 25, 2014].
- Hockaday, L. a, et al. (2012). Rapid 3D printing of anatomically accurate and mechanically heterogeneous aortic valve hydrogel scaffolds. *Biofabrication*, 4(3), 035005. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3676672&tool=pmcentrez&rendertype=abstract> [Accessed January 30, 2014].
- Jakab, K., et al. (2008). Tissue engineering by self-assembly of cells printed into topologically defined structures. *Tissue engineering. Part A*, 14(3), 413–421. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/18333793> [Accessed January 30, 2014].
- Jun, H.-W., & West, J. L. (2005). Endothelialization of microporous YIGSR/PEG-modified polyurethaneurea. *Tissue engineering*, 11(7–8), 1133–1140. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16144449>.
- Kanchanawong, P., et al. (2010). Nanoscale architecture of integrin-based cell adhesions. *Nature*, 468(7323), 580–584. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3046339&tool=pmcentrez&rendertype=abstract> [Accessed May 26, 2014].
- Kane, R. S., et al. (1999). Patterning proteins and cells using soft lithography. *Biomaterials*, 20(23–24), 2363–2376. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10614942> [Accessed June 20, 2014].
- Kim, S., et al. (2013). Engineering of functional, perfusable 3D microvascular networks on a chip. *Lab on a chip*, 13(8), 1489–1500. Available at: <http://pubs.rsc.org/EN/content/articlehtml/2013/lc/c3lc41320a> [Accessed June 6, 2014].
- King, K. R., et al. (2004). *Biodegradable Microfluidics*. *Advanced Materials*, 16(22), 2007–2012. Available at: <http://doi.wiley.com/10.1002/adma.200306522> [Accessed February 8, 2014].
- Kolesky, D. B., et al. (2014). 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. *Advanced materials (Deerfield Beach, Fla.)*, 26(19), 3124–3130. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24550124> [Accessed June 26, 2014].

- Lee, S.-H., Moon, J. J., & West, J. L. (2008). Three-dimensional micropatterning of bioactive hydrogels via two-photon laser scanning photolithography for guided 3D cell migration. *Biomaterials*, 29(20), 2962–2968. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/18433863> [Accessed February 28, 2014].
- Lin, H., et al. (2013). Application of visible light-based projection stereolithography for live cell-scaffold fabrication with designed architecture. *Biomaterials*, 34(2), 331–339. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3612429&tool=pmcentrez&rendertype=abstract> [Accessed February 28, 2014].
- Tsang, V. Liu, et al. (2007). Fabrication of 3D hepatic tissues by additive photopatterning of cellular hydrogels. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 21(3), 790–801. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17197384> [Accessed May 27, 2014].
- Liu, V., & Bhatia, S. (2002). Three-dimensional photopatterning of hydrogels containing living cells. *Biomedical microdevices*, 4(4), 257–266. Available at: <http://link.springer.com/article/10.1023/A:1020932105236> [Accessed June 17, 2014].
- McGuigan, A. P., & Sefton, M. V. (2007). The influence of biomaterials on endothelial cell thrombogenicity. *Biomaterials*, 28(16), 2547–2571. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20300848> [Accessed July 1, 2014].
- Miller, J. S., et al. (2012). Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues. *Nature materials*, 11(9), 768–774. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3586565&tool=pmcentrez&rendertype=abstract> [Accessed January 22, 2014].
- Miller, J. S. (2014). The billion cell construct: will three-dimensional printing get us there? *PLoS biology*, 12(6), e1001882. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24937565> [Accessed July 1, 2014].
- Murray, C. D. (1926). The Physiological Principle of Minimum Work: I. The Vascular System and the Cost of Blood Volume. *Proceedings of the National Academy of Sciences of the United States of America*, 12(3), 207–214. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1084489&tool=pmcentrez&rendertype=abstract> [Accessed June 25, 2014].
- Nakamura, M., et al. (2008). Ink Jet Three-Dimensional Digital Fabrication for Biological Tissue Manufacturing : Analysis of Alginate Microgel Beads Produced by Ink Jet Droplets for Three. *Journal of Imaging Science and Technology*, 52(6), 1–6.
- Nguyen, D.-H. T., et al. (2013). Biomimetic model to reconstitute angiogenic sprouting morphogenesis in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, 110(17), 6712–6717. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3637738&tool=pmcentrez&rendertype=abstract> [Accessed June 2, 2014].
- Nichol, J. W., et al. (2010). Cell-laden microengineered gelatin methacrylate hydrogels. *Biomaterials*, 31(21), 5536–5544. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2878615&tool=pmcentrez&rendertype=abstract> [Accessed February 20, 2014].
- Nishiyama, Y., et al. (2009). Development of a three-dimensional bioprinter: construction of cell supporting structures using hydrogel and state-of-the-art inkjet technology. *Journal of biomechanical engineering*, 131(3), 035001. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19154078> [Accessed February 28, 2014].
- Norotte, C., et al. (2009). Scaffold-free vascular tissue engineering using bioprinting. *Biomaterials*, 30(30), 5910–5917. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2748110&tool=pmcentrez&rendertype=abstract> [Accessed January 23, 2014].
- Ovsianikov, A., et al. (2008). Two-photon polymerization technique for microfabrication of CAD-designed 3D scaffolds from commercially available photosensitive materials, 443–449.
- Pham, Q. P., Sharma, U., & Mikos, A. G. (2006). Electrospun poly(epsilon-caprolactone) microfiber and multi-layer nanofiber/microfiber scaffolds: characterization of scaffolds and measurement of cellular infiltration. *Biomacromolecules*, 7(10), 2796–2805. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17025355>.
- Shaner, N. C., Steinbach, P. A., & Tsien, R. Y. (2005). A guide to choosing fluorescent proteins. *Nature methods*, 2(12), 905–909. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16299475> [Accessed May 23, 2014].

- Sherman, T. F. (1981). On connecting large vessels to small. The meaning of Murray's law. *The Journal of general physiology*, 78(4), 431–453. Available at: <http://www.jgp.org/cgi/doi/10.1085/jgp.78.4.431> [Accessed July 1, 2014].
- Skardal, A., et al. (2012). Bioprinted amniotic fluid-derived stem cells accelerate healing of large skin wounds. *Stem cells translational medicine*, 1(11), 792–802. Available at: <http://europepmc.org/articles/PMC3659666> [Accessed June 16, 2014].
- Skardal, A., Zhang, J., & Prestwich, G. D. (2010). Bioprinting vessel-like constructs using hyaluronan hydrogels crosslinked with tetrahedral polyethylene glycol tetracrylates. *Biomaterials*, 31(24), 6173–6181. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20546891> [Accessed February 28, 2014].
- Suri, S., et al. (2011). Solid freeform fabrication of designer scaffolds of hyaluronic acid for nerve tissue engineering. *Biomedical microdevices*, 13(6), 983–993. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21773726> [Accessed May 6, 2014].
- Truskett, V. N., & Watts, M. P. C. (2006). Trends in imprint lithography for biological applications. *Trends in biotechnology*, 24(7), 312–317. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16759722> [Accessed June 4, 2014].
- Vozzi, G., et al. (2003). Fabrication of PLGA scaffolds using soft lithography and microsyringe deposition. *Biomaterials*, 24(14), 2533–2540. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0142961203000528> [Accessed June 5, 2014].
- Whitesides, G. M., et al. (2001). Soft lithography in biology and biochemistry. *Annual review of biomedical engineering*, 3, 335–373. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11447067> [Accessed June 25, 2014].
- Wu, W., et al. (2010). Direct-write assembly of biomimetic microvascular networks for efficient fluid transport. *Soft Matter*, 6(4), 739. Available at: <http://xlink.rsc.org/?DOI=b918436h> [Accessed June 25, 2014].
- Xu, T., et al. (2008). Characterization of Cell Constructs Generated With Inkjet Printing Technology Using In Vivo Magnetic Resonance Imaging. *Journal of Manufacturing Science and Engineering*, 130(2), 021013. Available at: <http://manufacturingscience.asmedigitalcollection.asme.org/article.aspx?articleid=1452028> [Accessed February 28, 2014].
- Yannas, I. V., et al. (1982). Wound tissue can utilize a polymeric template to synthesize a functional extension of skin. *Science (New York, N.Y.)*, 215(4529), 174–176. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/7031899> [Accessed July 2, 2014].
- Zheng, Y., et al. (2012). In vitro microvessels for the study of angiogenesis and thrombosis. *Proceedings of the National Academy of Sciences of the United States of America*, 109(24), 9342–9347. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3386137&tool=pmcentrez&rendertype=abstract> [Accessed February 20, 2014].

CRANIOFACIAL AND DENTAL TISSUE

11

Michael Larsen¹, Ruchi Mishra², Michael Miller³ and David Dean⁴

¹*Plastic Surgeon & Reconstructive Hand Surgeon, Arizona Center for Hand to Shoulder Surgery, Phoenix, AZ, United States*

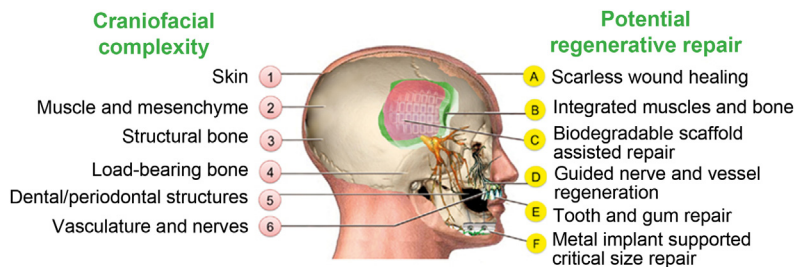
²*Former Faculty at National Institute of Technology, Raipur, Chhattisgarh, India* ³*Plastic and Reconstructive Surgeon, Banner MD Anderson Cancer Center, Gilbert, AZ, United States* ⁴*Department of Materials Science and Engineering; Department of Plastic and Reconstructive Surgery, The Ohio State University, Columbus, OH, United States*

11.1 INTRODUCTION

Craniofacial and dental structures are complex tissues that perform vital functions, such as seeing, hearing, breathing, chewing, tasting, speaking, and protecting the brain and eyes (Costello et al., 2010). Standard-of-care craniofacial and dental regenerative medicine consists largely of musculoskeletal reconstructive techniques analogous to orthopedic and general surgical reconstructive techniques, with special attention to the complex 3D structure (Figure 11.1) (Sanchez-Lara and Warburton, 2012). Most of these procedures are aimed at restoring function (e.g., mastication) and structure in an aesthetically appropriate reconstruction. What has changed over the past decade is the expected role that tissue engineering has and will have in regenerative medicine. This is illustrated by the conceptual move away from research in whole organ regeneration to structure- and defect-specific “regenerative medicine,” which includes elements of grafting, bone and dental substitute materials, and off-the-shelf or custom devices. Initial research success in all three of these areas is being translated to the repair of particular tissue deficits with a focus on the restoration of function through the augmentation of standard-of-care procedures. Many if not most reconstructive procedures continue to involve transplantation and alloplastic hardware or tissue substitutes.

Tissue engineering remains a scientific activity that has the potential to improve therapies for specific indications. However, tissue engineering has yet to contribute to the craniofacial therapies involving bone, glands, sense organs, joints, muscles, or dental tissues. Careful study of growth factors and progenitor cells in restoring failed craniofacial tissues is now done mostly in parallel to research into surgical therapies, material research, and mass market or patient-specific device research. All of this research is aimed at improving the treatment of craniofacial patients who suffer from traumatic injuries, cancer, or congenital deformity.

Custom devices do not yet involve biological components; thus, there is no true “bioprinting” However, there is initial use of growth factors, such as BMP-2, in some craniofacial therapies. There is much use of nanotechnology in the development of biomaterials for these implants and other surgical devices. A new wave of developments is occurring in developing biomaterials with a wide range of properties that can be rendered in 3D printers. It is likely that those materials will eventually be used as resorbable models, or scaffolds, for tissue engineering applications that utilize craniofacially relevant stem cells and growth factors.

**FIGURE 11.1**

Current craniofacial regenerative procedures.

(Figure 11.1 from Sanchez-Lara et al., 2011).

The next section of this chapter will look at the range of craniofacial therapies, third is a look at research into craniofacial and dental regenerative medicine, fourth is a look at current research into bone tissue engineering, and finally we state our conclusions from this review of craniofacial and dental tissue bioprinting and nanotechnology.

11.2 CLINICAL NEED FOR CRANIOFACIAL AND DENTAL REGENERATIVE MEDICINE

The craniofacial region is composed of a complex interconnection of multiple organ systems. The structure consists of a musculoskeletal framework that is both intricate enough to compliment the function of the other systems as well as adequately robust to provide protection to the delicate components therein. The craniofacial region is the beginning of the respiratory and alimentary tracts, functioning to warm and humidify air and commence the digestion of food in these respective systems. This area is also the sensory headquarters of the body, with all sensory modalities being manifest here; more particularly, the four special sensory systems (visual, auditory, olfactory, and gustatory) exclusively operate here. Stimuli from the environment are received here and are even processed here. The signals received by the sensory organs are then transmitted nearby to the most vital of organs, the brain. Thus, the craniofacial region is the central interface between the individual and their environment; it is also responsible for sending communicative signals by embodying voice into speech, expressing emotions, disclosing identity, displaying aesthetics, and cueing gender, race, and age. With the craniofacial region playing so many key functions, defects and deformities of this area can severely affect an individual's quality of life, leading to both significant physical impairments as well as psychological sequelae. Indeed, it has been shown that patients who have undergone resection of tumors of the head and neck actually more readily adjust to their dysfunction than to their disfigurement (Dropkin, 1999; Gamba et al., 1992). For all these reasons, reconstruction and restoration of craniofacial defects demands state-of-the-art techniques and advanced materials.

11.2.1 MAJOR DIAGNOSES AND CAUSES

Defects and deformities of the craniofacial region arise from dental disease, trauma, aging, cancer, and congenital malformations. Each of these will presently be discussed.

11.2.1.1 Dental Disease

Statistics show that 100% of adults have dental caries, and it is estimated that 15–20% of adults between the ages of 35–44 suffer from severe periodontal disease that will result in tooth loss. Dental disease is cumulative with age, resulting in 30% of people aged 65–74 becoming fully edentulous. The mandible and maxilla of the edentulous naturally atrophy with time, becoming much thinner and more susceptible to fracture.

11.2.1.2 Trauma

The primary cause of death in people under 40 years of age is trauma. Approximately 25,000 people per year require surgery to fix maxillofacial trauma in the United States. Maxillofacial trauma results from assault, motor vehicle collisions, falls, and sporting accidents. The relative incidence of these causes has been shifting. With increasing widespread use of airbags and seatbelts, the number and severity of maxillofacial fractures from motor vehicle collisions has been decreasing. On the other hand, as the population is aging and the elderly are seeking active lifestyles, the number of fractures from falls is trending up (Martinez et al., 2014).

11.2.1.3 Aging

As people age, the collagen framework loosens, the dermis thins, there is a cumulative solar elastosis, and muscles and bones of the face thin and atrophy. These result in an inferomedial descent of the soft tissue and the stigmata of aging including wrinkles, furrows, jowls, decreased dental show, the downward tilt of the corners of the mouth, and sunken cheeks.

11.2.1.4 Cancer

In the United States more than 40,000 people are diagnosed with oral and pharyngeal cancers each year. (The Oral Cancer Foundation; <http://oralcancerfoundation.org/facts/>). An additional 3.5 million people are diagnosed with a new skin cancer (basal cell, squamous cell, or melanoma). A majority of these occur in the highly sun-exposed craniofacial region. The ablation of craniofacial malignancies often requires resection of multiple surrounding tissue types (skin, subcutaneous fat, bone, and mucosa), ensuring adequate margins but leaving the patient with exposed bone, vessels, and nerves and defects that cannot be closed primarily. Radiation treatments further complicate wound healing by compromising the local vasculature.

11.2.1.5 Congenital

In the U.S. approximately 38,000 infants undergo surgery each year for the treatment of congenital defects. One in 1000–2000 infants are born with sporadic or inherited craniosynostosis (fusion of cranial plates), which can lead to a malformed cranium, intracranial hypertension, and restricted brain growth. An additional 1:1000 children are born with cleft lip and palate. Certain syndromes can result in hypoplasia of the maxilla (i.e., Apert's or Crouzon's) or mandible (i.e., Treacher Collins or Pierre Robin Sequence) and airway compromise. Furthermore, many are born with microtia or contour irregularities which they find aesthetically displeasing (CDC, 2008).

11.2.2 STANDARD-OF-CARE PROCEDURES

The standard of care for these craniofacial defects and deformities, with the exception of teeth, has historically centered around manipulating and transplanting the patient's own tissues. Autogenous tissue has the characteristics of an ideal biomaterial: biocompatible, nontoxic, nonallergenic,

Table 11.1 Autograft and allograft characteristics.

Bone graft	Structural strength	Osteoconduction	Osteoinduction	Osteogenesis
Autograft				
Cancellous	No	+++	+++	+++
Cortical	+++	++	++	++
Allograft				
Cancellous				
Frozen	No	++	+	No
Freeze-dried	No	++	+	No
Cortical				
Frozen	+++	+	No	No
Freeze-dried	+	+	No	No

noninflammatory, mechanically reliable, resistant to microorganism growth, and provides permanency and consistently reproducible results (Dickinson et al., 2011). Autogenous bone has the additional properties of being osteoconductive (framework conducive to new bone growth), osteoinductive (stimulates osteoprogenitor cells to differentiate and form new bone), and osteogenic (contains osteoblasts) (Tables 11.1 and 11.2). Treatment strategy is largely determined by the size of the defect and the tissues involved. The tissue categories discussed here are teeth, bone and cartilage, and soft tissue.

11.2.2.1 Teeth

Humans have been replacing lost teeth for thousands of years, as evidenced by archeological discoveries of mandibles with implants of carved bamboo, precious metal pegs, and shaped seashells. In the modern era, titanium implants have been used since the 1960s when Dr. Branemark first coined the term “osseointegration.” Today, implants are made from surgical grade 5 titanium (Ti-6Al-4V) (de Lavos-Valereto et al., 2002). These implants are crowned with materials such as glass-ceramics, zirconia, and alumina, all of which perform very well (Oilo et al., 2014), demonstrating appropriate wear of opposing natural crown surfaces (Kim et al., 2012b). Unfixed dentures or bridges represent less-involved treatment options. Teeth that are chipped or have defects can be bonded using resins, while misshapen or discolored incisors can be etched on their anterior surface and bonded to veneers. Thus, for the most part, routine dental defects have been solved by biomaterial implants and prostheses; yet, solutions are still needed for reconstruction in compromised conditions, such as when there is insufficient bone or mucosa or poor healing capabilities (Joos, 2009).

11.2.2.2 Bone and Cartilage

Simple craniofacial fractures (whether caused by trauma or therapeutically made by the surgeon), with favorable mechanical forces, merely need to be splinted (e.g., interdental wiring). If the fracture pattern is complicated or is subjected to forces that cause splaying, rotation, or

Table 11.2 Osteoconductive scaffolds.

Type	Graft	Osteoconduction	Osteoinduction	Osteogenesis	Advantages
Bone	Autograft	3	2	2	“Gold standard”
	Allograft	3	1	0	Availability in many forms
Biomaterials	DBM	1	2	0	Supplies osteoinductive BMPs, bone graft extender
	Collagen	2	0	0	Good as delivery vehicle system
Ceramics	TCP, hydroxyapatite	1	0	0	Biocompatible
	Calcium phosphate cement (CPC)	1	0	0	Some initial structural support
Composite grafts	-TCP/BMA composite	3	2	2	Ample supply
	BMP/synthetic	—	3	—	Potentially limitless supply

Score: 0 (none) to 3 (excellent). DBM: demineralised bone matrix, TCP: tricalcium phosphate, BMA: bone marrow aspirate, BMP: bone morphogenetic protein.

movement, the bones are fixated with screws and plates to avoid malunion, malocclusion, and fibrous (rather than bony) healing. Likewise, when a bone defect is large enough (critical-size or larger), it will heal by fibrous scar formation. In critical-size defects, a nonvascularized bone graft from the iliac crest, tibial plateau, outer table of the calvarium, or olecranon can be employed to fill the gap. Allografts (e.g., demineralized freeze-dried bone), xenografts (coral), metals (titanium), and alloplasts (e.g., hydroxyapatite, methylmethacrylate) are also occasionally used (see [Section 11.4.1](#)); however, caution is employed because of the risks of nonunion, infection, and extrusion. When a defect >6 cm is encountered or the wound is compromised (i.e., irradiated tissues), then a vascularized bone graft of radius, fibula, rib, or iliac crest is used ([Franklin et al., 1980](#); [Song et al., 1982](#); [Taylor, 1983](#); [Taylor et al., 1975, 1979](#)). Large maxillary defects are often filled with an obturator (a removable prosthetic) or a vascularized soft tissue flap; however, soft tissue is not ideal since it can result in asymmetry and contour abnormalities, and will not be able to accept osseointegrated implants for dental reconstruction ([Hanasono et al., 2010](#)). Furthermore, autogenous bone or soft tissue grafts have significant drawbacks: limited supply; inaccuracy in the 3D shaping and inset; and their need for increased operative time, larger surgical teams, more equipment, and extra operative site with its complications ([Ling and Peng, 2012](#)).

Patients with craniofacial hypoplasia are treated with distraction osteogenesis, where osteotomies (bone fractures) are iatrogenically made of the involved bones, and a device is applied that will distract

the bone segments apart 1 mm/day. This rate is slow enough to allow bone to form in the intervening gap. This is continued until the desired bone lengthening is achieved.

The cranial bones of craniosynostosis patients are cut apart, repositioned, and then fixated according to the principles mentioned (usually with plates and screws with or without bone graft).

Ear deformities (e.g. microtia, atresia) can be reconstructed by harvesting autogenous rib cartilage, carving it into the shape of an ear, and implanting it under the skin. However, the reconstructed ear often lacks symmetry with, and the aesthetic definition of, the contralateral ear. Thus, recent data reported by Reinisch et al. showing optimal aesthetic outcomes and minimal complications with a medpor (high-density porous polyethylene) ear implant could lead to medpor becoming the gold standard treatment for this deformity (Reinisch and Li, 2014).

11.2.2.3 Soft Tissue

The treatment paradigm for soft tissue defects has traditionally adhered to the reconstructive ladder, where simple and more local solutions are used if possible; but when these options are nonviable, the surgeon steps up to a feasible but more complex solution (Figure 11.2). Many surgeons have also begun expanding their personal reconstructive ladder to include skin substitutes, such as Integra (Integra NeuroSciences, Plainsboro, NJ), which is a bilayered construct that contains an inner layer consisting of a collagen/glycosaminoglycan matrix that acts as a scaffold for cellular ingrowth and dermal regeneration.

Some early products for facial augmentation were made of expanded polytetrafluoroethylene (ePTFE, W.L. Gore & Associates Inc., Newark, DE; PTFE is best known as Teflon, Dupont Co., Wilmington, DE). These products were largely abandoned because of extremely high complication

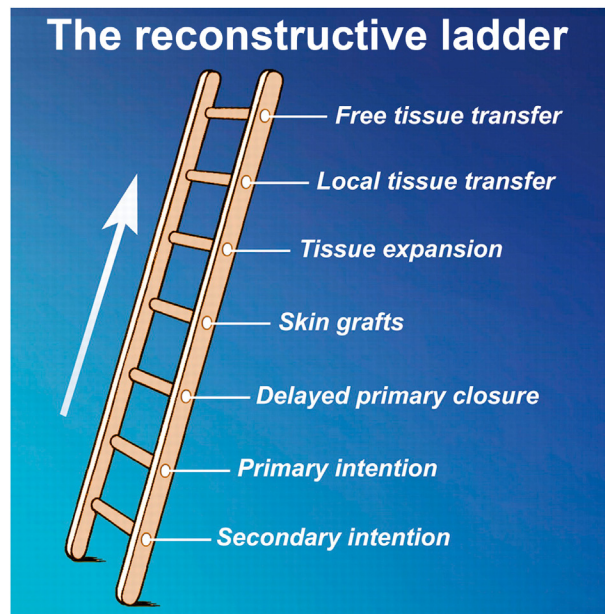


FIGURE 11.2

The reconstructive ladder.

rates, including palpability, migration, shrinkage, and extrusion (Maloney et al., 2012; Truswell, 2007). Injectable fillers have since come to the market and have rapidly become one of the most common cosmetic procedures, with 2.2 million injections in the U.S. last year (13% most recent annual increase) (2014, ASPS, 2014). Various materials are used as fillers: hyaluronic acid, calcium hydroxylapatite, poly-L-lactic acid, silicone, and polymethylmethacrylate (Figure 11.3) (de Vries and Geertsma, 2013). Autologous fat injections have also been used with success (Barton et al., 2007). In addition, newer ePTFE implants have been developed with a softer, more natural quality, and a higher porosity (allowing more cellular ingrowth), that have both led to low complication rates (Niamtu, 2006).

While autografts are currently the gold standard for many craniofacial therapies, it can be seen that the standard of care is shifting. The significant disadvantages of autografts demand the development of new biomaterials, further advances in craniofacial regenerative medicine, and a continued shift in the standard of care.

Filler	What it is made of	What it treats	Avg. length of effectiveness
Juvéderm® Ultra	Hyaluronic Acid	- Folds around the mouth - Temples - Cheeks - Lips	6–18 months
Juvéderm® Ultra Plus	Hyaluronic Acid	- Folds around the mouth - Temples - Cheeks - Lips	6–18 months
Restylane®	Hyaluronic Acid	- Folds around the mouth - Under eyes - Scars - Nose - Lips	6–31 months
Perlane®	Hyaluronic Acid	- Folds around the mouth - Cheeks - Lips	6–18 months
Radiesse®	Calcium Hydroxylapatite	- Moderate to deep facial wrinkles and folds - Under eyes - Cheeks - Nose	Up to 1–2 Years
Sculptra®	Poly-L-lactic Acid	- Moderate to deep facial wrinkles and folds - Cheeks	Up to 1–2 Years
ArteFill®	Collagen and Polymethyl Methacrylate	- Moderate to deep facial wrinkles and folds - Cheeks	Up to 5+ Years

FIGURE 11.3

Injectable fillers.

11.3 CRANIOFACIAL AND DENTAL REGENERATIVE MEDICINE RESEARCH

Despite the great need, there is very little penetration of tissue engineering, especially additive manufacturing or cell printing for tissue engineering, in standard-of-care craniofacial surgical and/or dental therapeutic procedures that repair critical size defects (Schmitz and Hollinger, 1986). However, the significant use in these specialties of bone and tooth substitute materials, especially in regard to the additive manufacture of patient-specific medical implants, may eventually forge a link between tissue engineering and craniofacial and dental care.

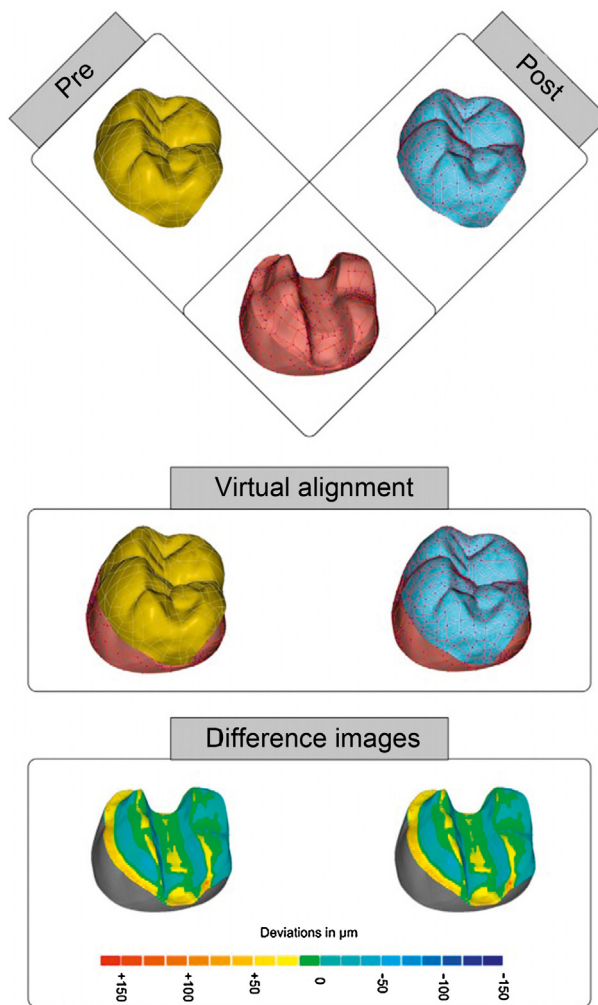
11.3.1 NOVEL MATERIALS

Implants and surgical reconstruction hardware for the craniofacial and dental regions have typically used the same materials as elsewhere in the body. However, there are many advanced materials that are used fairly exclusively in the craniofacial region. Resorbable Lactosorb (Lorenz [Biomet], Jacksonville, FL), which is 82% poly(-L lactic acid) and 18% poly(glycolic acid) is one such example (Biomet microfixation, 2010). These plates and screws resorb by hydrolysis within a year, allowing uninhibited growth of the bones and obviating the need for a secondary procedure to remove the hardware. These plates have particular application in softer bones, such as in the orbit and in infants. Solid-state hyaluronic acid in the form of a thread is another advanced material used in the craniofacial region. This promising new product allows for more controlled wrinkle effacement and soft tissue augmentation (Franklin, 2014). Nitinol-shape memory metals are used in orthodontic wire; these wires are superelastic at body temperature. As mentioned previously, titanium dental implant bone screws and abutments are very successful. However, there is a great deal of innovation with other alloys, zirconia, and coatings designed to improve osseointegration. Single crown or fixed prosthesis implant dental systems are currently more expensive than nonfixed systems (e.g., traditional dentures), but they also provide for better mechanical function, stimulation, and maintenance of oral cavity muscles and skeletal structures. However, the most exciting and cutting-edge application of such advanced biomaterials is in their use in the additive manufacture (3D printing) of patient-specific implants in teeth, craniofacial bone, and the temporomandibular joint.

11.3.2 TEETH

The Sirona CEREC1 (Chairside Economical Restorations of Esthetic Ceramics), the first system to combine digital scanning of the teeth, and computer-aided design (CAD) of prosthetic crowns, appeared in 1985. Most of these crown restorations are currently prepared in the prosthodontist's office or by a nearby service with computerized milling devices. These systems are sufficiently accurate (Figure 11.4), therefore there seems to be no benefit in terms of quality or price at this time in moving to more advanced fabrication strategies (Schaefer et al., 2013).

Ng et al., (2014) noted that it is only recently that restorations prepared in this way have become more economical and perform better than traditional manual methods. Given the success of these post-and-crown systems, the challenge for oral health has reverted back to the issue of sufficient bone bulk to support dental posts. This is especially challenging in edentulous

**FIGURE 11.4**

This comparison of image-based crown design (pre) and rendered crown (post) shows high accuracy of these milled crowns. (Schaefer et al., 2013).

individuals (Cho and Raigrodski, 2014). Dental implants and single crown or fixed prostheses may require autologous and/or alloplastic bone graft materials. This depends on the size of the alveolar cavity left following dental extraction. It is unclear at this time whether these expensive fixed prostheses will become standard of care under the United States' Affordable Care Act in nontraumatic situations (O'Brien, 2014).

11.3.3 BONE

In cases of craniofacial bone shortage, there are no tissue engineered therapies. Alloplastic materials have been explored. Polymethylmethacrylate (PMMA) is the most common material used followed by grade 5 surgical titanium (Ti-6Al-4V). These materials have been used to make custom cranioplasties (skull plates) for more than a decade (Dean et al., 2003a). Cranial plates are now commonly 3D printed directly from CAD files in PEEK (Lethaus et al., 2012) with a newer material, PEKK (Baum, 2013), having more appropriate material properties for a bone substitute implant. If alloplastic materials are used, a key consideration is how well they transfer strain to and from the surrounding skull (Figure 11.5). Creating adaptive, rather than stress-shielding or stress-concentrating, reconstructions is a major consideration in the treatment of mandibular segmental defects if permanent surgical grade 5 titanium fixation is used (Zoumalan et al., 2009). The topological optimization (Figure 11.6) of very stiff implant components in craniofacial implants has been studied by Sutradhar et al., (2010).

Mandibular segmental defects are now commonly being repaired with fibular bone grafts where fibular and mandibular cutting guides are provided and Ti-6Al-4V immobilization plates are present. This saves operating room time, and enhances reproducibility and precision, which in turn aids boney union and prevents fibrous ingrowth of the defect space (Wang et al., 2013; Saad et al.,

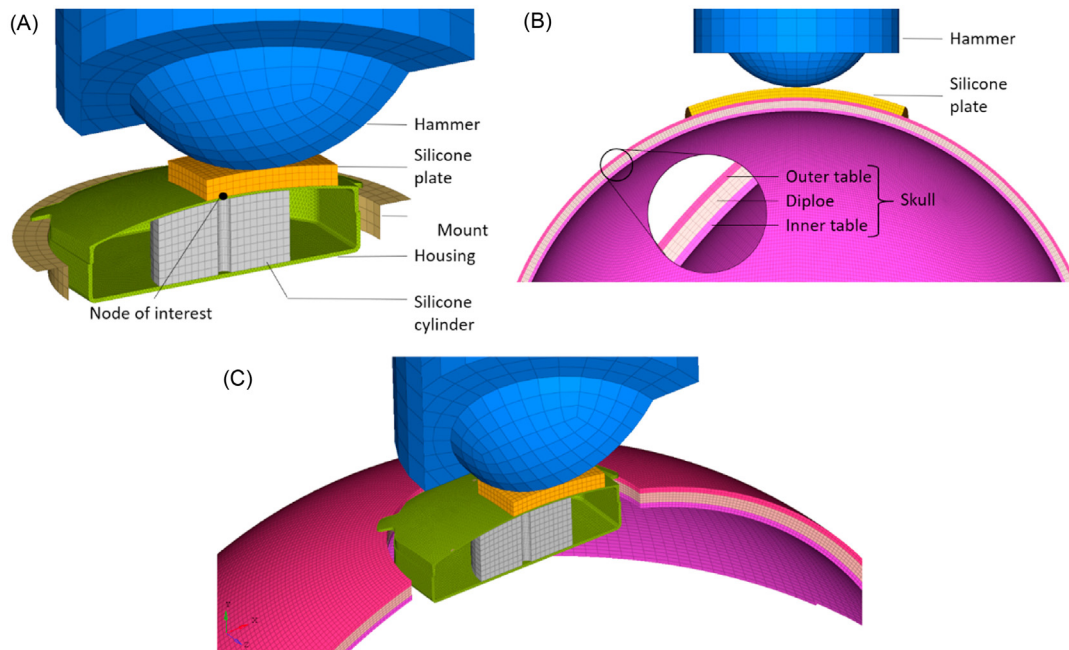
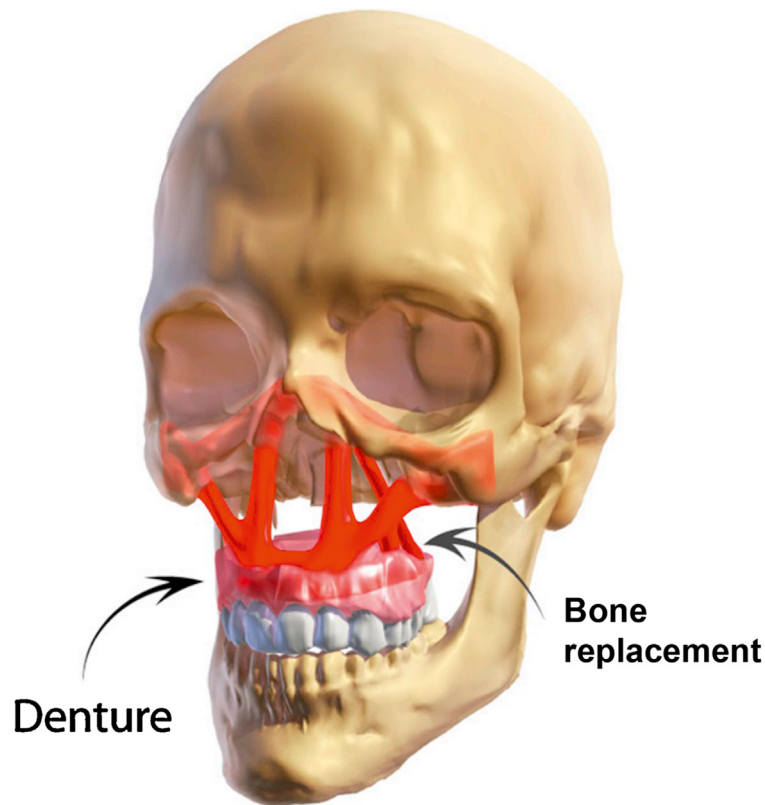


FIGURE 11.5

Custom cranial implant material properties: A. Mechanical testing device. B. Three layers or tables of normal bone versus homogenous structure in the implant. C. Applying compressive force to the implant.

(Siegel et al., 2021)

**FIGURE 11.6**

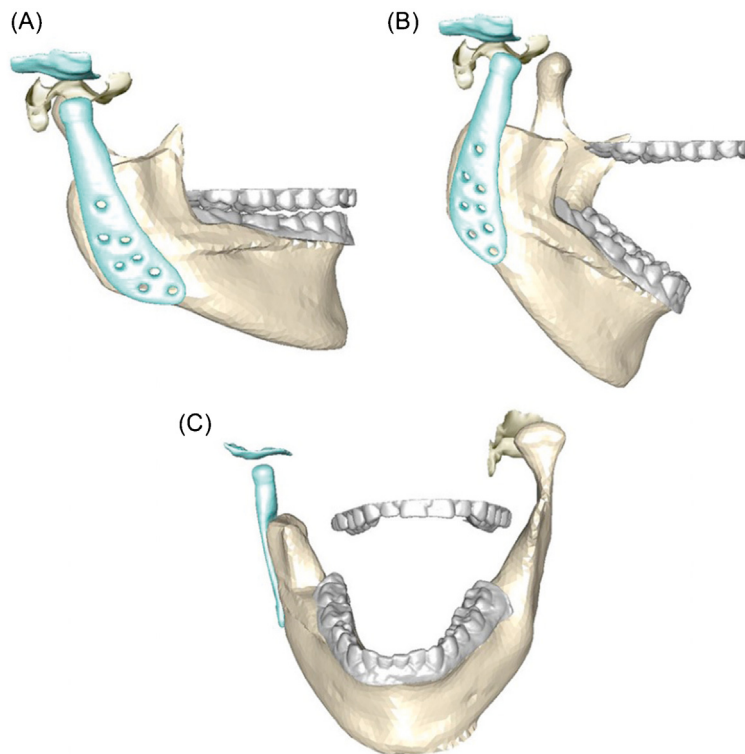
Topological Optimization: Optimization of stress-strain trajectories suggests the need for appropriate strength in the displayed struts.

(Sutradhar et al., 2010).

2013). Others have presented their experience with the replacement of most (Zhou et al., 2010) to all (van Kroonenburgh et al., 2012) of the mandible with 3D printed titanium prosthetics. The long-term outcomes of such cases remain to be seen; it is known that the use of titanium or acrylic instead of bone graft or Guided Tissue Regeneration (Park and Wang, 2007) devices directly under the gingivae may risk dehiscence (Chiapasco and Zaniboni, 2009).

11.3.4 TEMPOROMANDIBULAR JOINT

Involvement of the temporomandibular joint (TMJ) in the repair of segmental mandibular defects elevates the complexity. The TMJ includes the head of the mandible, articular disc, or the articular tubercle (of the zygomatic arch). This joint has been cited as one of the most active joints in the body (Guarda-Nardini et al., 2008). Early attempts at total joint replacement involving Teflon joint surfaces were associated with foreign-body giant cell reactions resulting in damage to the surrounding host structures and an unstable joint (Abramowicz et al., 2008). Recent experience has found a

**FIGURE 11.7**

Mandibular positions during chewing following unilateral total TMJ replacement: (A) mouth closed (lateral view). (B) Maximum gape (lateral view). (C) Maximum gape (frontal view).

(From: Leiggener et al., 2012).

wide variety of stable implant configurations, with good performance by patient-specific devices (Mercuri, 2012) and using metal-on-polyethylene TMJ replacements (Figure 11.7) with stable, long-term outcomes (Leiggener et al., 2012).

11.4 BONE TISSUE ENGINEERING STRATEGIES

Tissue engineering strategies have the potential to augment traditional restorative approaches that utilize transplanted tissues and bone substitute materials. Some key considerations in the development of tissue-engineered strategies for craniofacial/dental tissue are as follows: (1) are there any stem cells that can be used; (2) can aesthetic demands be met; (3) can tissue-engineered constructs be used as part of a composite strategy; (4) can adequate vascularization demands be met; and (5) can the high risk of infection in the oral and nasal cavities be managed (Moioli et al., 2007). These aspects have also been discussed in detail by (Mikos et al., 2006) (Table 11.3). Craniofacial tissue-engineered strategies are likely

Table 11.3 Functional device considerations and requirements for oral and craniofacial bone tissue engineering devices (based on Table 1 of Mikos et al., 2006).

Tissue engineering scaffold strategy considerations	Tissue engineering scaffold requirements
Infection	Is the wound bed where the device is to be placed 1. contaminated prior to implantation? 2. directly under either a mucous membrane or the superficial layer of the overlying skin, thereby risking erosion and exposure of a deep space to the oral cavity, nasal or paranasal sinuses, or, in the case of the skin, continuously to the external environment?
Wound healing capability	Is there a nearby blood supply that will articulate well (i.e., including microvasculature and capillary level) with the construct?
Potential scaffold requirements	1. Placement of scaffold 2. Can scaffold be used to deliver cells? 3. Are mechanical properties appropriate for wound healing and bone formation? 4. Does scaffold degrade slowly enough to guide tissue formation but quickly enough so as not to be a barrier to tissue formation and/or vascularization?
Cell signaling	1. If cell signaling molecules (e.g., growth factors) are included in scaffold, can an appropriate dose be provided and released or made available when needed? 2. Are there multiple cell signaling molecules with different dose and temporal release requirements?
Validation	Are there clinically relevant 1. small animal models to test biocompatibility beyond cytotoxicity testing? 2. large animal models to test ability to regenerate Oral and/or Craniofacial bone?
Clinical requirements	1. Is animal model testing of full range of defect sizes, especially large size, that are to be treated (i.e., is it reflective of full range of intended clinical use)? 2. If cells and growth factors are used, can enough of one or both be delivered to bring about healing?
Fabrication and craniofacial and oral region-specific challenges	1. Is there a way to fabricate device with personalized defect external shape and any needed internal morphology (e.g., pores)? 2. Tissue engineering dental/periodontal ligament, periodontal ligament/bone, tendon/bone, bone/cartilage, or neurovascular bundle interface 3. Regeneration of patient-specific enamel, dentin, cementum, pulp, and neurovascular structures of teeth

to be required to regenerate more than one tissue phenotype at the same time (Moioli et al., 2007; Rahaman and Mao, 2005).

Bone tissue engineering-based strategies for craniofacial/dental tissue regeneration can be divided broadly into three main categories based on the scaffold, cellular, or growth factor component that plays the major role in the expected regeneration phenomenon. Bioreactor preculture or surface

functionalization of these implants may also be used to limit the attachment of cells to a specific type and to help those cells commit to play a role after implantation by having a functioning tissue type (e.g., by producing extracellular matrix) or by prevascularizing the tissue via coculture with endothelial cells (Temple et al., 2013).

11.4.1 SCAFFOLDS

Artificial scaffold materials for craniofacial/dental tissue regeneration may act like the extracellular matrix of the desired tissue by supporting cell attachment, proliferation, and differentiation of host or seeded cells in the presence of biological cues. The scaffolds are commonly resorbable and are expected to resorb at a rate similar to that of new bone formation (Hutmacher, 2000). These scaffolds can be used in different forms such as porous 3D solids (Dean et al., 2012; Kim et al., 2011), nanofibers (Gupte and Ma, 2012; Li et al., 2014), cement/putty (Kim et al., 2012a), among others. Various types of scaffolds used for craniofacial/dental tissue regeneration can be divided into three main classes: ceramic/bioactive glasses, natural/synthetic polymers, and composites (Salgado et al., 2004). Illustrations of all three scaffold material classes follow:

11.4.1.1 Ceramic/bioactive Glasses

The ceramic/bioactive glasses are a class of biomaterials made up of inorganic materials having high compressive strength, but low tensile strength (brittle) characteristics. Calcium phosphate-based materials have been extensively used for craniofacial/dental tissue engineering in the form of injectable calcium phosphate cement (CPC) (Chen et al., 2014; Thein-Han et al., 2012) or implanted scaffolds (Chan et al., 2009). Hydroxyapatite in the form of BoneSource[®] cement (Stryker, Kalamazoo, MI) has also been used for applications in craniofacial tissue engineering and reconstruction (Costantino et al., 1992; Friedman et al., 1998). Hydroxyapatite scaffolds prepared by additive manufacturing (a.k.a. 3D printing) have been studied for their repair capability in minipig mandibular defects for 6 and 18 weeks (Hollister et al., 2005). Bioactive glasses have also been studied as biomaterials for craniofacial reconstruction (Cho and Gosain, 2004; Gosain, 2004). One exciting new product which promises to be quite adaptive is being developed by Filardo et al. Using a novel bioceramization process, they have been able to turn wood into a strong, highly porous product with an elastic modulus that approaches that of bone (Filardo et al., 2014).

11.4.1.2 Natural/synthetic Polymers

Biopolymers can be prepared synthetically or from naturally occurring sources. Polymers tend to be more flexible or ductile in nature than ceramics, although they usually have less compressive strength than ceramics. Biopolymers derived from natural sources used for craniofacial regeneration include collagen (Narotam et al., 2007), fibrin, hyaluronic acid (Kretlow et al., 2009), alginate, silk (Ye et al., 2011), and chitosan (Canter et al., 2010). Biopolymers of synthetic origin studied in bone tissue engineering applications include poly(propylene fumarate) (PPF) (Dean et al., 2012, 2003b), polylactic acid (PLA) (Di Bella et al., 2008), polyglycolic acid (PGA), poly(lactic-co-glycolic acid) (PLGA) (Kaigler et al., 2006), polycaprolactone (PCL) (Schantz et al., 2003), and polyethylene glycol (Terella et al., 2010). Most of these biopolymers have also been used for dental tissue engineering (Horst et al., 2012).

11.4.1.3 Composites

Blending ceramic, polymeric, and possibly metal or graft tissue components may result in an implant with the desired material properties that is at least partially resorbable. Polymers and ceramics are most likely to mimic bone extracellular matrix which in itself is a blend of inorganic (hydroxyapatite) and organic (collagen type I) components. Some of the composites that have been used in various combinations for craniofacial/dental tissue engineering are: beta-tricalcium phosphate, collagen, and autologous bone fragments (Kishimoto et al., 2006); polycaprolactone (PCL)-tricalcium phosphate (TCP) (Probst et al., 2010); biocomposite cryogels (Mishra et al., 2014; Mishra and Kumar, 2014); and chitosan and inorganic phosphates (Stephan et al., 2010).

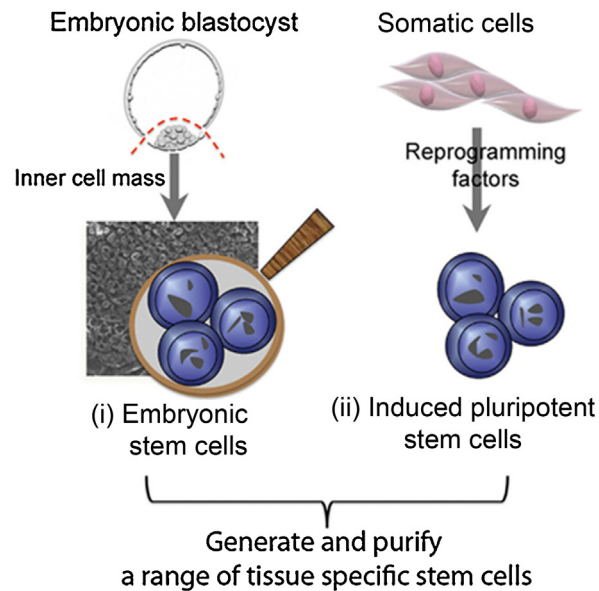
11.4.2 GROWTH FACTORS

Bone morphogenetic proteins (BMPs) are the most studied signaling molecules related to bone maturation. Twenty types of BMPs encoded by the human genome have been identified (Nakashima and Reddi, 2003). Out of these, the BMPs that have been most widely studied for craniofacial/dental regeneration are BMP-2 and BMP-7. These BMPs have been studied for cranial regeneration (Guda et al., 2014) and alveolar tissue as well as oral or dental implant osseointegration (Dunn et al., 2005; Wikesjo et al., 2005). GMP (Good Manufacturing Practice) level recombinant human bone morphogenetic protein-2 (rhBMP-2) provided by R&D Systems (Minneapolis, MN) and Akron Biotechnology (Boca Raton, FL) has been approved for bone applications such as interbody spinal fusion, open tibial fractures, and sinus and alveolar ridge augmentations. It is available commercially in the INFUSE[®] Bone Graft product (McKay et al., 2007). Infuse is composed of rhBMP-2, which is present on an absorbable collagen sponge (ACS) functioning as a carrier. For certain craniofacial/dental applications such as for defects related with extraction sockets, INFUSE[®] Bone Graft received FDA approval for sinus augmentations and localized alveolar ridge augmentations in 2007 (McKay et al., 2007). Problematic sequelae have been reported for approved and “off-label” use of BMP-2 such as hematoma, seroma, and swelling, *among others*. The safety of BMP-2 has been discussed (Epstein, 2013; Neovius et al., 2013; Smucker et al., 2006). Other growth factors involved in vasculogenesis, such as vascular endothelial growth factors (VEGFs), are also used for craniomaxillofacial regeneration because bone is a highly vascular tissue and vasculogenesis is expected to facilitate bone regeneration (Kaigler et al., 2006). Section 11.4.5 discusses bioreactor administration of growth factors. The use of bioreactors avoids in vivo use and therefore any unintended pleiotropic effects such as those observed with BMP-2. Where growth factor pharmacokinetics and sensitivity are well understood, there are a variety of delivery mechanisms (Vo et al., 2012).

11.4.3 CELL-BASED THERAPIES

The cells used for cell-based therapies for craniofacial/dental tissue engineering are mostly stem cells (Krebsbach and Robey, 2002). Stem cells are attractive candidates for tissue engineering because they are clonogenic and capable of self-renewal, therefore small populations of stem cells can proliferate to provide the desired cell number within a shorter time span. They have the potential to differentiate into different cell types (Rosa et al., 2012). Some of the cells that have been used for craniofacial/dental tissue engineering (Figure 11.8) are mesenchymal stem cells (MSCs) derived from

(A) Pluripotent stem cells



(B) Adult stem cells

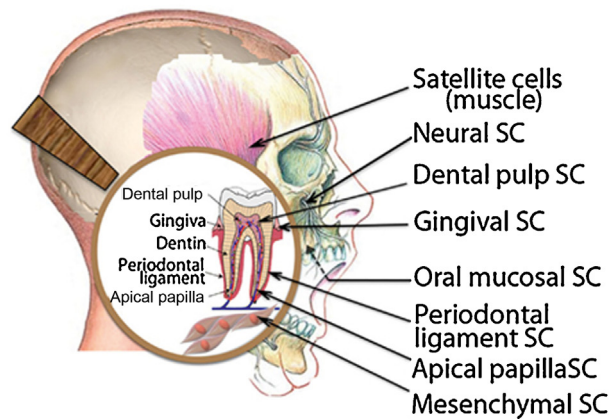


FIGURE 11.8

Specialized tissues where regenerative scaffolds may benefit from preculturing and stem cell differentiation, perhaps in a bioreactor.

(Figure 11.1 from: Sanchez-Lara et al., 2011).

bone marrow or adipocytes (Marra and Rubin, 2012), dental pulp stem cells (DPSCs), differentiated osteoblasts, perivascular cells, stem cells from exfoliated deciduous teeth (SHEDs), stem cells from apical papilla (SCAPs), periodontal ligament stem cells (PDLSCs), and dental follicle precursor cells (DFPCs) (Bhatt and Le Anh, 2009; Costello et al., 2010; Machado et al., 2012; Risbud and Shapiro, 2005). Dental tissue derived mesenchymal stem cells is a relatively new area of research that may find a wider role in craniofacial/dental repair (Yang et al., 2014). However, it is still not determined whether MSCs from bone marrow, adipocytes, or dental sources will be more favorable for stem cell-based repair of craniofacial/dental tissue (Estrela et al., 2011; Mao et al., 2006; Marra and Rubin, 2012). Recently, constructs developed by cells alone (scaffoldless) have been studied as a potential cell source in dental tissue engineering (Syed-Picard et al., 2014).

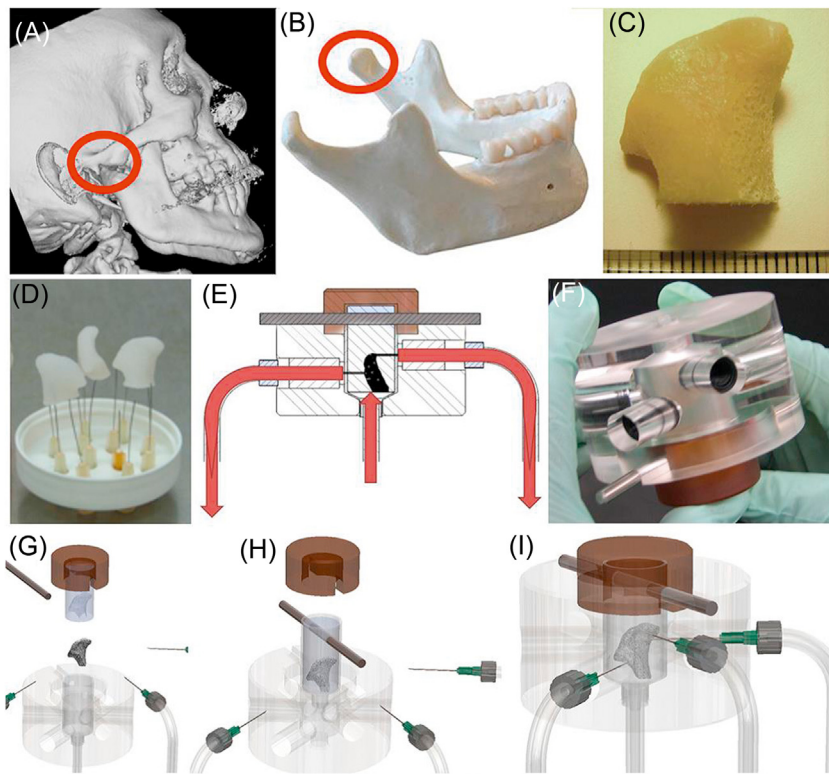
11.4.4 NEW CRANIOFACIAL TISSUES

Two of the more difficult craniofacial tissues are now being explored to see if stem cells can be used in regenerative therapies. The first tissue is retina, where retinal stem cells have received attention given their role in producing sight (Hambright et al., 2012). This work is currently focused on elucidating the mechanisms of retinal tissue repair and treatments for degenerative diseases. The regeneration of sight may be somewhat further off (Yip, 2014). The second tissue is oral mucosa, a highly specialized tissue that is at a high risk of failure and infection following masticatory and dental reconstructive surgery. Good integration between oral mucosa, underlying reconstructed structures, and around percutaneous implants is the goal of this work (Jones and Klein, 2013).

11.4.5 BIOREACTORS

Preculturing of cells inside a bioreactor, with and without shear stress (i.e. from flow or external compression or tension), nutrients, and signaling molecules has been studied as a way of preculturing cells. One of the advantages of preculturing is the production of sufficient cells (starting from low cell densities) to coat the scaffold and to bring about their maturation to facilitate the host's recognition and integration of the implant as osseous tissue (Mikos et al., 2006). Such a biomaterial represents a tissue-engineered graft (Wallace et al., 2014). This is especially important in clinical settings where it may be difficult to carry out rigorous capture and handling of autologous cells in a distributed, as opposed to a centralized, fashion. It may be especially difficult to characterize the potency (i.e., ability to produce the desired tissue) of all sources of autologous cells.

Bioreactor culturing of well-characterized cells may also offer the advantages of acclimatizing cells to the scaffolds prior to implantation, uniform distribution of oxygen and nutrients throughout the clinical-size bone substitutes, and controlling the amount, nature, and duration of mechanical stimulation applied to seeded cells (de Peppo, 2014). Bioreactor culture of cells has been found to be favorable for craniofacial applications such as the generation of the mandibular condyle (Alhadlaq and Mao, 2003), auricular cartilage (Alhadlaq et al., 2004), the entire TMJ condylar bone (Grayson et al., 2010), and dental applications such as periodontal tissue regeneration (Jin et al., 2003). The protocol for the bioreactor cultivation of cells in tissue engineering of a TMJ by (Grayson et al., 2010) is demonstrated in Figure 11.9.

**FIGURE 11.9**

Bioreactor cultivation for the development of human temporomandibular joint (TMJ) condyles: (A–D) scaffold development; (D) image showing scaffold complexity; (E) The seeding of hMSCs in a bioreactor; (F) photograph of perfusion bioreactor; (G–I) images representing bioreactor assembly.

(From Grayson *et al.*, 2010).

11.5 Conclusions

Bioprinting and nanotechnology research into craniofacial and dental regenerative medicine therapies has utilized growth factors; however, the use of resorbable scaffolds and autologous or banked cells—the remaining elements of tissue engineering—have not yet made their way to the clinic. Craniofacial and dental bioprinting and nanotechnology are concentrated on patient-specific inert devices. This is a large industry that has recently shown dramatic improvements in therapeutic efficacy and safety. There is a revolution occurring in developing 3D printable materials that cover the range of material properties needed for patient-specific implants to perform mechanically like, indeed in tandem with, the tissues to which they are attached. As advances are made in bone, retina, and other areas of craniofacial and dental tissue engineering, they are likely to be incorporated into standard-of-care therapies, unless they are novel enough to shift the current paradigm. Bioprinting and regenerative medicine promise true quality

care by facilitating the physician in “doing the right thing, at the right time, in the right way, for the right person—and having the best possible results.” However, the current paradigm is relatively new; therefore, it remains to be seen whether the benefits of patient-specific medicine will be made available throughout society through mechanisms like the Affordable Care Act in the United States.

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References

- Abramowicz, S., Dolwick, M. F., Lewis, S. B., & Dolce, C. (2008). Temporomandibular joint reconstruction after failed teflon-proplast implant: case report and literature review. *Int J Oral Maxillofac Surg*, 37, 763–767.
- Alhadlaq, A., & Mao, J. J. (2003). Tissue-engineered neogenesis of human-shaped mandibular condyle from rat mesenchymal stem cells. *J Dent Res*, 82, 951–956.
- Alhadlaq, A., Elisseeff, J. H., Hong, L., Williams, C. G., Caplan, A. I., Sharma, B., Kopher, R. A., Tomkoria, S., Lennon, D. P., Lopez, A., & Mao, J. J. (2004). Adult stem cell driven genesis of human-shaped articular condyle. *Ann Biomed Eng*, 32, 911–923.
- ASPS. 2014. Plastic Surgery Procedures Continue Steady Growth in U.S. | ASPS.
- Barton, F. E., Jr., Carruthers, J., Coleman, S., & Graivier, M. (2007). The role of toxins and fillers in perioral rejuvenation. *Aesthetic surgery journal / the American Society for Aesthetic Plastic surgery*, 27, 632–640.
- Baum, S. 2013. Skull implant produced with 3-D printing technology could shake up orthopedics industry. Medcity News.
- Bhatt, A., & Anh, D. Le (2009). Craniofacial tissue regeneration: where are we? *J Calif Dent Assoc*, 37, 799–803.
- Biomet microfixation. 2010.
- Canter, H. I., Vargel, I., Korkusuz, P., Oner, F., Gungorduk, D. B., Cil, B., Karabulut, E., Sargon, M. F., & Erk, Y. (2010). Effect of use of slow release of bone morphogenetic protein-2 and transforming growth factor-Beta-2 in a chitosan gel matrix on cranial bone graft survival in experimental cranial critical size defect model. *Ann Plast Surg*, 64, 342–350.
- Chan, W. D., Perinpanayagam, H., Goldberg, H. A., Hunter, G. K., Dixon, S. J., Santos, G. C., Jr., & Rizkalla, A. S. (2009). Tissue engineering scaffolds for the regeneration of craniofacial bone. *J Can Dent Assoc*, 75, 373–377.
- Chen, W., Thein-Han, W., Weir, M. D., Chen, Q., & Xu, H. H. (2014). Prevascularization of biofunctional calcium phosphate cement for dental and craniofacial repairs. *Dent Mater*, 30, 535–544.
- Chiapasco, M., & Zaniboni, M. (2009). Clinical outcomes of GBR procedures to correct peri-implant dehiscences and fenestrations: a systematic review. *Clin Oral Implants Res*, 20(Suppl 4), 113–123.
- Cho, Y. R., & Gosain, A. K. (2004). Biomaterials in craniofacial reconstruction. *Clin Plast Surg*, 31, 377–385, v.
- Cho, Y., & Raigrodski, A. J. (2014). The rehabilitation of an edentulous mandible with a CAD/CAM zirconia framework and heat-pressed lithium disilicate ceramic crowns: A clinical report. *J Prosthet Dent* 111(6), 443–447.
- Costantino, P. D., Friedman, C. D., Jones, K., Chow, L. C., & Sisson, G. A. (1992). Experimental hydroxyapatite cement cranioplasty. *Plast Reconstr Surg*, 90, 174–185, discussion 186-191.
- Costello, B. J., Shah, G., Kumta, P., & Sfeir, C. S. (2010). Regenerative medicine for craniomaxillofacial surgery. *Oral Maxillofac Surg Clin North Am*, 22, 33–42.

- de Lavos-Valereto, I. C., Deboni, M. C., Azambuja, N., Jr., & Marques, M. M. (2002). Evaluation of the titanium Ti-6Al-7Nb alloy with and without plasma-sprayed hydroxyapatite coating on growth and viability of cultured osteoblast-like cells. *J Periodontol*, 73, 900–905.
- de Vries, C. G. J. C. A., & Geertsma, R. E. (2013). Clinical data on injectable tissue fillers: a review. *Expert Review of Medical Devices*, 10, 835–853.
- Dean, D., Jonathan, W., Siblani, A., Wang, M. O., Kim, K., Mikos, A. G., & Fisher, J. P. (2012). Continuous Digital Light Processing (cDLP): Highly Accurate Additive Manufacturing of Tissue Engineered Bone Scaffolds. *Virtual Phys Prototyp*, 7, 13–24.
- Dean, D., Min, K. J., & Bond, A. (2003a). Computer aided design of large-format prefabricated cranial plates. *J Craniofac Surg*, 14, 819–832.
- Dean, D., Topham, N. S., Meneghetti, S. C., Wolfe, M. S., Jepsen, K., He, S., Chen, J. E., Fisher, J. P., Cooke, M., Rinnac, C., & Mikos, A. G. (2003b). Poly(propylene fumarate) and poly(DL-lactic-co-glycolic acid) as scaffold materials for solid and foam-coated composite tissue-engineered constructs for cranial reconstruction. *Tissue Eng*, 9, 495–504.
- Di Bella, C., Farlie, P., & Penington, A. J. (2008). Bone regeneration in a rabbit critical-sized skull defect using autologous adipose-derived cells. *Tissue Eng Part A*, 14, 483–490.
- Dickinson, B. P., Roy, I., & Lesavoy, M. A. (2011). Temporalis Fascia for Lip Augmentation. *Annals of Plastic Surgery*, 66, 114–117.
- Dropkin, M. J. (1999). Body image and quality of life after head and neck cancer surgery. *Cancer Practice*, 7, 309–313.
- Dunn, C. A., Jin, Q., Taba, M., Jr., Franceschi, R. T., Rutherford, R. Bruce, & Giannobile, W. V. (2005). BMP gene delivery for alveolar bone engineering at dental implant defects. *Mol Ther*, 11, 294–299.
- Epstein, N. E. (2013). Complications due to the use of BMP/INFUSE in spine surgery: The evidence continues to mount. *Surg Neurol Int*, 4, S343–352.
- Estrela, C., Alencar, A. H., Kitten, G. T., Vencio, E. F., & Gava, E. (2011). Mesenchymal stem cells in the dental tissues: perspectives for tissue regeneration. *Braz Dent J*, 22, 91–98.
- Filardo, G., Kon, E., Tampieri, A., Cabezas-Rodriguez, R., Di Martino, A., Fini, M., Giavaresi, G., Lelli, M., Martinez-Fernandez, J., Martini, L., Ramirez-Rico, J., Salamanna, F., Sandri, M., Sprio, S., & Marcacci, M. (2014). *New Bio-Ceramization Processes Applied to Vegetable Hierarchical Structures for Bone Regeneration: An Experimental Model in Sheep*. *Tissue Engineering Part A*, 20, 763–773.
- Franklin, R. 2014. Solid Hyaluronic Acid Tailored to Fix Fine Wrinkles.
- Franklin, J. D., Shack, R. B., Stone, J. D., Madden, J. J., & Lynch, J. B. (1980). SINGLE-STAGE RECONSTRUCTION OF MANDIBULAR AND SOFT-TISSUE DEFECTS USING A FREE OSTEOCUTANEOUS GROIN FLAP. *American Journal of Surgery*, 140, 492–498.
- Friedman, C. D., Costantino, P. D., Takagi, S., & Chow, L. C. (1998). BoneSource hydroxyapatite cement: a novel biomaterial for craniofacial skeletal tissue engineering and reconstruction. *J Biomed Mater Res*, 43, 428–432.
- Gamba, A., Romano, M., Grosso, I. M., Tamburini, M., Cantu, G., Molinari, R., & Ventafridda, V. (1992). PSYCHOSOCIAL ADJUSTMENT OF PATIENTS SURGICALLY TREATED FOR HEAD AND NECK-CANCER. *Head and Neck-Journal for the Sciences and Specialties of the Head and Neck*, 14, 218–223.
- Gosain, A. K. (2004). Bioactive glass for bone replacement in craniomaxillofacial reconstruction. *Plast Reconstr Surg*, 114, 590–593.
- Grayson, W. L., Frohlich, M., Yeager, K., Bhumiratana, S., Chan, M. E., Cannizzaro, C., Wan, L. Q., Liu, X. S., Guo, X. E., & Vunjak-Novakovic, G. (2010). *Engineering anatomically shaped human bone grafts*. *Proc Natl Acad Sci U S A*, 107, 3299–3304.
- Guarda-Nardini, L., Manfredini, D., & Ferronato, G. (2008). Temporomandibular joint total replacement prosthesis: current knowledge and considerations for the future. *Int J Oral Maxillofac Surg*, 37, 103–110.
- Gupte, M. J., & Ma, P. X. (2012). Nanofibrous scaffolds for dental and craniofacial applications. *J Dent Res*, 91, 227–234.

- Hambright, D., Park, K. Y., Brooks, M., McKay, R., Swaroop, A., & Nasonkin, I. O. (2012). Long-term survival and differentiation of retinal neurons derived from human embryonic stem cell lines in un-immunosuppressed mouse retina. *Mol Vis*, 18, 920–936.
- Hanasono, M. M., Skoracki, R. J., & Yu, P. R. (2010). A Prospective Study of Donor-Site Morbidity after Anterolateral Thigh Fasciocutaneous and Myocutaneous Free Flap Harvest in 220 Patients. *Plastic and Reconstructive Surgery*, 125, 209–214.
- Hollister, S. J., Lin, C. Y., Saito, E., Schek, R. D., Taboas, J. M., Williams, J. M., Partee, B., Flanagan, C. L., Diggs, A., Wilke, E. N., Van Lenthe, G. H., Muller, R., Wirtz, T., Das, S., Feinberg, S. E., & Krebsbach, P. H. (2005). Engineering craniofacial scaffolds. *Orthod Craniofac Res*, 8, 162–173.
- Horst, O. V., Chavez, M. G., Jheon, A. H., Desai, T., & Klein, O. D. (2012). Stem cell and biomaterials research in dental tissue engineering and regeneration. *Dent Clin North Am*, 56, 495–520.
- Hutmacher, D. W. (2000). Scaffolds in tissue engineering bone and cartilage. *Biomaterials*, 21, 2529–2543.
- Jin, Q. M., Zhao, M., Webb, S. A., Berry, J. E., Somerman, M. J., & Giannobile, W. V. (2003). *Cementum engineering with three-dimensional polymer scaffolds*. *J Biomed Mater Res A*, 67, 54–60.
- Jones, K. B., & Klein, O. D. (2013). Oral epithelial stem cells in tissue maintenance and disease: the first steps in a long journey. *Int J Oral Sci*, 5, 121–129.
- Joos, U. 2009. *Tissue Engineering Strategies in Dental Implantology*.
- Kaigler, D., Wang, Z., Horger, K., Mooney, D. J., & Krebsbach, P. H. (2006). VEGF scaffolds enhance angiogenesis and bone regeneration in irradiated osseous defects. *J Bone Miner Res*, 21, 735–744.
- Kim, J., McBride, S., Fulmer, M., Harten, R., Garza, Z., Dean, D. D., Sylvia, V. L., Doll, B., Wolfgang, T. L., Gruskin, E., & Hollinger, J. O. (2012a). Fiber-reinforced calcium phosphate cement formulations for cranioplasty applications: a 52-week duration preclinical rabbit calvaria study. *J Biomed Mater Res B Appl Biomater*, 100, 1170–1178.
- Kim, K., Dean, D., Wallace, J., Breithaupt, R., Mikos, A. G., & Fisher, J. P. (2011). The influence of stereolithographic scaffold architecture and composition on osteogenic signal expression with rat bone marrow stromal cells. *Biomaterials*, 32, 3750–3763.
- Kim, M. J., Oh, S. H., Kim, J. H., Ju, S. W., Seo, D. G., Jun, S. H., Ahn, J. S., & Ryu, J. J. (2012b). Wear evaluation of the human enamel opposing different Y-TZP dental ceramics and other porcelains. *J Dent*, 40, 979–988.
- Kishimoto, M., Kanemaru, S., Yamashita, M., Nakamura, T., Tamura, Y., Tamaki, H., Omori, K., & Ito, J. (2006). Cranial bone regeneration using a composite scaffold of Beta-tricalcium phosphate, collagen, and autologous bone fragments. *Laryngoscope*, 116, 212–216.
- Krebsbach, P. H., & Robey, P. G. (2002). Dental and skeletal stem cells: potential cellular therapeutics for craniofacial regeneration. *J Dent Educ*, 66, 766–773.
- Kretlow, J. D., Young, S., Klouda, L., Wong, M., & Mikos, A. G. (2009). Injectable biomaterials for regenerating complex craniofacial tissues. *Adv Mater*, 21, 3368–3393.
- Leiggener, C. S., Erni, S., & Gallo, L. M. (2012). Novel approach to the study of jaw kinematics in an alloplastic TMJ reconstruction. *Int J Oral Maxillofac Surg*, 41, 1041–1045.
- Lethaus, B., Safi, Y., ter Laak-Poort, M., Kloss-Brandstatter, A., Banki, F., Robbenmenke, C., Steinseifer, U., & Kessler, P. (2012). Cranioplasty with customized titanium and PEEK implants in a mechanical stress model. *J Neurotrauma*, 29, 1077–1083.
- Li, G., Zhang, T., Li, M., Fu, N., Fu, Y., Ba, K., Deng, S., Jiang, Y., Hu, J., Peng, Q., & Lin, Y. (2014). Electrospun fibers for dental and craniofacial applications. *Curr Stem Cell Res Ther*, 9, 187–195.
- Ling, X. F., & Peng, X. (2012). What Is the Price to Pay for a Free Fibula Flap? A Systematic Review of Donor-Site Morbidity following Free Fibula Flap Surgery. *Plastic and Reconstructive Surgery*, 129, 657–674.
- Machado, E., Fernandes, M. H., & Pde, S. Gomes (2012). Dental stem cells for craniofacial tissue engineering. *Oral Surg Oral Med Oral Pathol Oral Radiol*, 113, 728–733.
- Maloney, B.P., W. Truswell, and S. R. Waldman. 2012. Lip Augmentation Discussion and Debate. *Facial Plastic Surgery Clinics of North America* 20(3):327–346.

- Mao, J. J., Giannobile, W. V., Helms, J. A., Hollister, S. J., Krebsbach, P. H., Longaker, M. T., & Shi, S. (2006). Craniofacial tissue engineering by stem cells. *J Dent Res*, 85, 966–979.
- de Peppo, G. M., Vunjak-Novakovic, G., & Marolt, D. (2014). Cultivation of Human Bone-Like Tissue from Pluripotent Stem Cell-Derived Osteogenic Progenitors in Perfusion Bioreactors. *Methods Mol Biol*.
- Marra, K. G., & Rubin, J. P. (2012). The potential of adipose-derived stem cells in craniofacial repair and regeneration. *Birth Defects Res C Embryo Today*, 96, 95–97.
- Martinez, A. Y., Como, J. J., Vacca, M., Nowak, M. J., Thomas, C. L., & Claridge, J. A. (2014). Trends in Maxillofacial Trauma: A Comparison of Two Cohorts of Patients at a Single Institution 20 Years Apart. *Journal of Oral and Maxillofacial Surgery*, 72, 750–754.
- McKay, W. F., Peckham, S. M., & Badura, J. M. (2007). A comprehensive clinical review of recombinant human bone morphogenetic protein-2 (INFUSE Bone Graft). *Int Orthop*, 31, 729–734.
- Mercuri, L. G. (2012). Alloplastic temporomandibular joint replacement: rationale for the use of custom devices. *Int J Oral Maxillofac Surg*, 41, 1033–1040.
- Mikos, A. G., Herring, S. W., Ochareon, P., Elisseeff, J., Lu, H. H., Kandel, R., Schoen, F. J., Toner, M., Mooney, D., Atala, A., Van Dyke, M. E., Kaplan, D., & Vunjak-Novakovic, G. (2006). Engineering complex tissues. *Tissue Eng*, 12, 3307–3339.
- Mishra, R., & Kumar, A. (2014). Osteocompatibility and osteoinductive potential of supermacroporous polyvinyl alcohol-TEOS-Agarose-CaCl₂ (PTAgC) biocomposite cryogels. *J Mater Sci Mater Med*, 25, 1327–1337.
- Mishra, R., Goel, S. K., Gupta, K. C., & Kumar, A. (2014). Biocomposite cryogels as tissue-engineered biomaterials for regeneration of critical-sized cranial bone defects. *Tissue Eng Part A*, 20, 751–762.
- Moioli, E. K., Clark, P. A., Xin, X., Lal, S., & Mao, J. J. (2007). Matrices and scaffolds for drug delivery in dental, oral and craniofacial tissue engineering. *Adv Drug Deliv Rev*, 59, 308–324.
- Nakashima, M., & Reddi, A. H. (2003). The application of bone morphogenetic proteins to dental tissue engineering. *Nat Biotechnol*, 21, 1025–1032.
- Narotam, P. K., Reddy, K., Fewer, D., Qiao, F., & Nathoo, N. (2007). Collagen matrix duraplasty for cranial and spinal surgery: a clinical and imaging study. *J Neurosurg*, 106, 45–51.
- Neovius, E., Lemberger, M., Docherty Skogh, A. C., Hilborn, J., & Engstrand, T. (2013). Alveolar bone healing accompanied by severe swelling in cleft children treated with bone morphogenetic protein-2 delivered by hydrogel. *J Plast Reconstr Aesthet Surg*, 66, 37–42.
- Ng, J., Ruse, D., & Wyatt, C. (2014). A comparison of the marginal fit of crowns fabricated with digital and conventional methods. *J Prosthet Dent*.
- Niamtu, J. (2006). Advanta ePTFE facial implants in cosmetic facial surgery. *Journal of Oral and Maxillofacial Surgery*, 64, 543–549.
- O'Brien, E. 2014. "Obamacare isn't good for your teeth: Health reform hasn't reduced adult dental costs." How to protect your teeth and wallet. Market Watch: The Wall Street Journal.
- Oilo, M., Hardang, A. D., Ulsund, A. H., & Gjerdet, N. R. (2014). Fractographic features of glass-ceramic and zirconia-based dental restorations fractured during clinical function. *Eur J Oral Sci*.
- Park, S. H., & Wang, H. L. (2007). Clinical significance of incision location on guided bone regeneration: human study. *J Periodontol*, 78, 47–51.
- Poukens, J., Lambrichts I, Beerens M, and M. P. 2012. Total Jaw Implant: The world's first 3D printed total jaw reconstruction.
- Probst, F. A., Hutmacher, D. W., Muller, D. F., Machens, H. G., & Schantz, J. T. (2010). [Calvarial reconstruction by customized bioactive implant]. *Handchir Mikrochir Plast Chir*, 42, 369–373.
- Rahaman, M. N., & Mao, J. J. (2005). Stem cell-based composite tissue constructs for regenerative medicine. *Biotechnol Bioeng*, 91, 261–284.

- Reinisch, J. F., & Li, W.-Y. (2014). Abstract 2: medpor ear reconstruction: a twenty-three year experience with 1042 ears. *Plastic and reconstructive surgery*, 133, 974–1974.
- Risbud, M. V., & Shapiro, I. M. (2005). Stem cells in craniofacial and dental tissue engineering. *Orthod Craniofac Res*, 8, 54–59.
- Rosa, V., Bona, A., Della, Cavalcanti, B. N., & Nor, J. E. (2012). Tissue engineering: from research to dental clinics. *Dent Mater*, 28, 341–348.
- Saad, A., Winters, R., Wise, M. W., Dupin, C. L., & Hilaire, H., St. (2013). Virtual surgical planning in complex composite maxillofacial reconstruction. *Plastic and Reconstructive Surgery*, 132, 626–633.
- Salgado, A. J., Coutinho, O. P., & Reis, R. L. (2004). Bone tissue engineering: state of the art and future trends. *Macromol Biosci*, 4, 743–765.
- Sanchez-Lara, P. A., & Warburton, D. (2012). Impact of stem cells in craniofacial regenerative medicine. *Front Physiol*, 3, 188.
- Sanchez-Lara, Pedro A., Hu, Zhao S., Ruchi, Bajpai S., Abdelhamid, Alaa I., & Warburton, David (2011). Impact of stem cells in craniofacial regenerative medicine. *Frontiers in Physiology*, 3, 188.
- Schaefer, O., Kuepper, H., Thompson, G. A., Cachovan, G., Hefti, A. F., & Guentsch, A. (2013). Effect of CNC-milling on the marginal and internal fit of dental ceramics: a pilot study. *Dent Mater*, 29, 851–858.
- Schantz, J. T., Hutmacher, D. W., Lam, C. X., Brinkmann, M., Wong, K. M., Lim, T. C., Chou, N., Guldberg, R. E., & Teoh, S. H. (2003). Repair of calvarial defects with customised tissue-engineered bone grafts II. *Evaluation of cellular efficiency and efficacy in vivo. Tissue Eng*, 9(Suppl 1), S127–139.
- Schmitz, J. P., & Hollinger, J. O. (1986). The critical size defect as an experimental model for craniomandibulofacial nonunions. *Clin Orthop Relat Res*, 299–308.
- Smucker, J. D., Rhee, J. M., Singh, K., Yoon, S. T., & Heller, J. G. (2006). Increased swelling complications associated with off-label usage of rhBMP-2 in the anterior cervical spine. *Spine (Phila Pa 1976)*, 31, 2813–2819.
- Song, R. Y., Gao, Y. Z., Song, Y. G., Yu, Y. S., & Song, Y. L. (1982). THE FOREARM FLAP. *Clinics in Plastic Surgery*, 9, 21–26.
- Stephan, S. J., Tholpady, S. S., Gross, B., Petrie-Aronin, C. E., Botchway, E. A., Nair, L. S., Ogle, R. C., & Park, S. S. (2010). Injectable tissue-engineered bone repair of a rat calvarial defect. *Laryngoscope*, 120, 895–901.
- Sutradhar, A., Paulino, G. H., Miller, M. J., & Nguyen, T. H. (2010). Topological optimization for designing patient-specific large craniofacial segmental bone replacements. *Proc Natl Acad Sci U S A*, 107, 13222–13227.
- Syed-Picard, F. N., Ray, H. L., Jr., Kumta, P. N., & Sfeir, C. (2014). Scaffoldless tissue-engineered dental pulp cell constructs for endodontic therapy. *J Dent Res*, 93, 250–255.
- Taylor, G. I. (1983). THE CURRENT STATUS OF FREE VASCULARIZED BONE-GRAFTS. *Clinics in Plastic Surgery*, 10, 185–209.
- Taylor, G. I., Miller, G. D. H., & Ham, F. J. (1975). FREE VASCULARIZED BONE GRAFT - CLINICAL EXTENSION OF MICROVASCULAR TECHNIQUES. *Plastic and Reconstructive Surgery*, 55, 533–544.
- Taylor, G. I., Townsend, P., & Corlett, R. (1979). SUPERIORITY OF THE DEEP CIRCUMFLEX ILIAC VESSELS AS THE SUPPLY FOR FREE GROIN FLAPS - CLINICAL-WORK. *Plastic and Reconstructive Surgery*, 64, 745–759.
- Temple, J. P., Yeager, K., Bhumiratana, S., Vunjak-Novakovic, G., & Grayson, W. L. (2014). Bioreactor Cultivation of Anatomically Shaped Human Bone Grafts. *Methods Mol Biol*.
- Terella, A., Mariner, P., Brown, N., Anseth, K., & Streubel, S. O. (2010). Repair of a calvarial defect with bio-factor and stem cell-embedded polyethylene glycol scaffold. *Arch Facial Plast Surg*, 12, 166–171.
- Thein-Han, W., Liu, J., & Xu, H. H. (2012). Calcium phosphate cement with biofunctional agents and stem cell seeding for dental and craniofacial bone repair. *Dent Mater*, 28, 1059–1070.

- Truswell, W. H. (2007). Using permanent implant materials for cosmetic enhancement of the perioral region. *Facial plastic surgery clinics of North America*, 15(4), 433–444, vi.
- Vo, T. N., Kasper, F. K., & Mikos, A. G. (2012). Strategies for controlled delivery of growth factors and cells for bone regeneration. *Adv Drug Deliv Rev*, 64, 1292–1309.
- Wang, L., Yoon, D. M., Spicer, P. P., Henslee, A. M., Scott, D. W., Wong, M. E., Kasper, F. K., & Mikos, A. G. (2013). Characterization of porous polymethylmethacrylate space maintainers for craniofacial reconstruction. *J Biomed Mater Res B Appl Biomater*, 101, 813–825.
- Wikesjo, U. M., Polimeni, G., & Qahash, M. (2005). Tissue engineering with recombinant human bone morphogenetic protein-2 for alveolar augmentation and oral implant osseointegration: experimental observations and clinical perspectives. *Clin Implant Dent Relat Res*, 7, 112–119.
- Yang, M., Zhang, H., & Gangolli, R. (2014). Advances of mesenchymal stem cells derived from bone marrow and dental tissue in craniofacial tissue engineering. *Curr Stem Cell Res Ther*, 9, 150–161.
- Ye, J. H., Xu, Y. J., Gao, J., Yan, S. G., Zhao, J., Tu, Q., Zhang, J., Duan, X. J., Sommer, C. A., Mostoslavsky, G., Kaplan, D. L., Wu, Y. N., Zhang, C. P., Wang, L., & Chen, J. (2011). Critical-size calvarial bone defects healing in a mouse model with silk scaffolds and SATB2-modified iPSCs. *Biomaterials*, 32, 5065–5076.
- Yip, H. K. (2014). Retinal stem cells and regeneration of vision system. *Anat Rec (Hoboken)*, 297, 137–160.
- Zhou, L. B., Shang, H. T., He, L. S., Bo, B., Liu, G. C., Liu, Y. P., & Zhao, J. L. (2010). Accurate reconstruction of discontinuous mandible using a reverse engineering/computer-aided design/rapid prototyping technique: a preliminary clinical study. *J Oral Maxillofac Surg*, 68, 2115–2121.
- Zoumalan, R. A., Hirsch, D. L., Levine, J. P., & Saadeh, P. B. (2009). Plating in microvascular reconstruction of the mandible: can fixation be too rigid? *J Craniofac Surg*, 20, 1451–1454.

3D Printing for Craniofacial Bone Regeneration

12

Naboneeta Sarkar^{1,2,*}, Yuxiao Zhou^{1,2,*} and Warren Grayson^{1,2,3,4,5}

¹*Department of Biomedical Engineering, School of Medicine, Johns Hopkins University, Baltimore, MD, United States*

²*Translational Tissue Engineering Center, School of Medicine, Johns Hopkins University, Baltimore, MD, United States*

³*Department of Materials Science & Engineering, Whiting School of Engineering, Johns Hopkins University, Baltimore, MD, United States*

⁴*Department of Chemical & Biomolecular Engineering, Whiting School of Engineering, Johns Hopkins University, Baltimore, MD, United States*

⁵*Institute for NanoBioTechnology (INBT), Johns Hopkins University School of Engineering, Baltimore, MD, United States*

12.1 INTRODUCTION

According to the Global Burden of Disease Study, there are about 7.5 million new facial fractures globally every year (Lalloo et al., 2019). The most common causes for craniofacial bone fractures are ground and sports accidents, congenital anomalies, surgeries, personal assaults, and falls (Allareddy, Allareddy, & Nalliah, 2011; Hardt & Kuttenger, 2010; Jin et al., 2018), and the most common types of injuries include maxillofacial, nasal bone, and orbital fractures (Allareddy et al., 2011; Jin et al., 2018; Yu et al., 2020). Unlike long bones, craniofacial bones have complicated anatomies and contribute to delicate functions such as breathing and mastication. Furthermore, craniofacial defects put patients at a higher risk for social and psychological stress. These factors make the treatment of critical-sized craniofacial defects generally more challenging than reconstructing other segmental bone defects.

The earliest written account of a craniofacial bone grafting procedure is recorded in the late 17th century when a canine bone was used for a successful restoration of a human cranial defect (De Boer, 1988). A wide range of biomaterials have since evolved to complement autografts, allografts, and xenografts (Oppenheimer, Tong, & Buchman, 2008; Thrivikraman, Athirasala, Twohig, Boda, & Bertassoni, 2017). To date, autografts (graft material transferred from one part of the body to another within the same individual) serve as the gold standard for craniofacial bone defects because they are histocompatible and do not trigger an immunogenic reaction (Salyer & Taylor, 1987). However, since autografts involve a second surgery in the patient's body, they have the drawbacks of limited supply of donor tissue, as well as, significant donor site morbidity or injury, deformity, invasive surgical risks including inflammation, bleeding, infection, and chronic pain. At present, the iliac crest is the most frequently used donor site for autologous grafting, since it provides an adequate harvest for cortical, cancellous, and cortico-cancellous bone depending upon the

*Equal contribution.

need (Sudhakar, Mohanty, & Singh, 2017). Apart from offering the highest concentration of osteo-progenitor cells, grafts harvested from the iliac crest also show less morbidity with a reasonable long-term success rate [$\sim 70\%$ for mandibular reconstruction from nonvascularized iliac bone (Elsalanty & Genecov, 2009)]. Since the early 1980s, calvarial grafts have grown in popularity in craniofacial reconstruction due to their slower resorption rate and suitable mechanical properties (Smolka, Eggensperger, Kollar, & Iizuka, 2005). Based on the positive results including low donor site morbidity and lower postsurgical complications, calvarial bone grafts are frequently utilized for the correction of congenital abnormalities, severe midfacial trauma, defects in the cranium, orbital walls, and zygoma (Jackson, Helden, & Marx, 1986).

While nonvascularized bone grafts provide better facial esthetics and contour restoration, it is not a treatment of choice for primary reconstruction or defects larger than 9 cm (Allsopp, Hunter-Smith, & Rozen, 2016). Vascularized grafts from fibula, iliac crest, scapula, and radius are ideal for large mandibular defect reconstruction since they are independent of blood and nutrient supply, which comes through the anastomosis of the vascular pedicle (Winters, Kraak, Oosterhuis, & de Kleuver, 2013). Apart from this major biological advantage, vascularized grafts also exhibit slower resorption rates, superior mechanical properties, preservation of cellular viability, and resistance to infection (Muramatsu, Hashimoto, Tominaga, & Taguchi, 2014). However, even with a success rate of 90% (Pogrel, Podlesh, Anthony, & Alexander, 1997), the vascularized grafts are mostly limited to mandibular defects because harvesting needs special surgical techniques which add significantly to the operation time and trauma to the patient.

Bone grafts harvested from genetically nonidentical donors of the same species are known as allografts. The use of allografts eliminates the risks of donor site morbidity, reduces surgery time, as well as provides an adequate supply of larger grafts (Griffin et al., 2015). However, infection, loss of structural integrity, and disease transmission are some of the major concerns associated with allograft reconstruction (Bostrom & Seigerman, 2005). Throughout history, xenografts or animal-derived bone tissue have been widely used to repair craniofacial defects, yet over time their use has been discontinued due to reports of poor osseointegration, immunological reaction, and host rejection. However, xenograft-derived scaffolds, typically from the bovine and porcine origin, which are chemically treated to retain inorganic material and remove organic elements became popular due to low cost, increased availability, and decreased disease transmission risk (Laurencin & El-Amin, 2008). Various commercially available xenograft-based products, such as Bio-Oss (deproteinized bovine bone), Gen-Os (porcine bone), Orthoss (bovine-derived bone graft), Bio-Gide (porcine type I and type III collagen), Neu-Coll [type I bovine dermal fibrillar collagen, hydroxyapatite (HA), and tricalcium phosphate (TCP)], and Osteograft (inorganic bovine bone mineral), are available for craniofacial bone reconstruction (Sheikh et al., 2017).

In recent years, developments of synthetic biocompatible materials and innovations in manufacturing technology opened up a myriad of novel scaffold choices for craniofacial bone grafts (Thrivikraman et al., 2017). A wide variety of materials including natural and synthetic polymers, bioceramics, composites, and metal are researched to be used for cost-effective biomedical purposes (Koons, Diba, & Mikos, 2020). The material selection depends on the loading expectation and biological need at the site of application. For craniofacial applications, bioceramic scaffolds are most commonly chosen, followed by metal implants and lastly by polymers (Maroulakos, Kamperos, Tayebi, Halazonetis, & Ren, 2019). In the past few decades, 3D printing revolutionized the manufacturing technology and soon its applications were being adapted within the biomedical

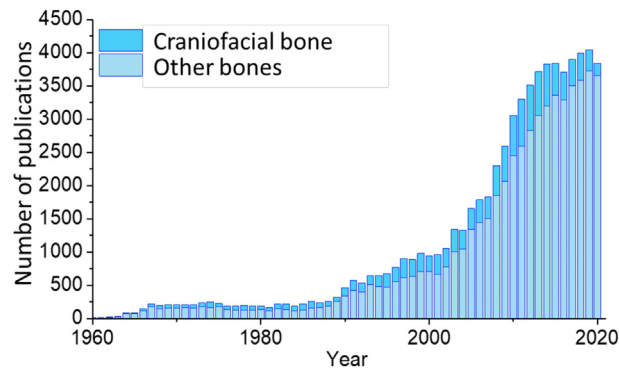


FIGURE 12.1

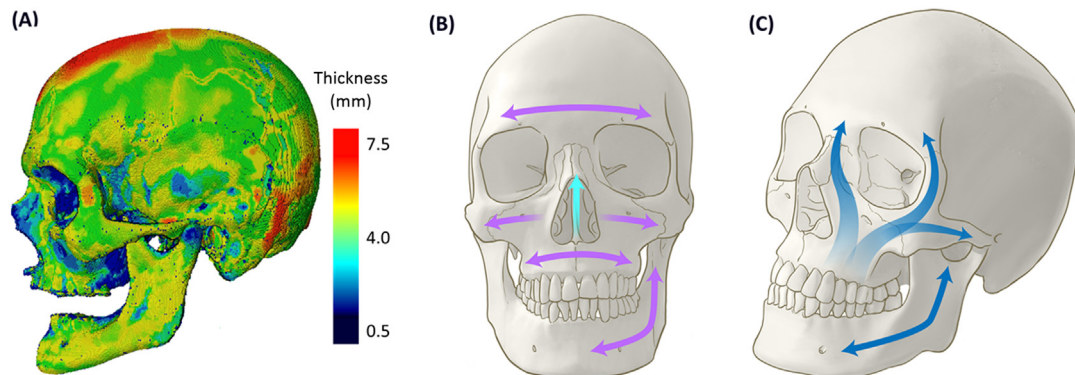
Growth of modern bone and craniofacial bone regeneration field. Number of published papers on bone regeneration (light blue) and craniofacial bone regeneration (blue) since 1960.

field to prepare surgical templates and synthetic graft. Compared to conventional grafts, 3D-printed craniofacial grafts offer an advantage by providing customized implants with a high degree of complexity to match specific defect shape and size. Among different 3D-printing technologies, laser printing is the most common approach to synthesize craniofacial grafts, followed by inkjet printing and microextrusion (Maroulakos et al., 2019). 3D-printed craniofacial implants have been mostly utilized to treat mandibular bone defects but have also been used for calvarial, maxillary, and nasal defect reconstruction. With the development of 3D-printing technique and stem cell-based regenerative medicine field, interest in applying these new techniques to craniofacial bone scaffold has grown exponentially over the last 20 years (Fig. 12.1). This chapter discusses the structure of craniofacial bone, traditional theories in craniofacial biomechanics, and how those impact the complexity in craniofacial scaffold design. Critical concerns and challenges in developing patient-specific scaffold and treatment plans will be discussed, followed by developments in 3D-printing techniques and materials as well as methods to enhance bone regeneration after transplantation.

12.2 ANATOMY AND MECHANICS OF CRANIOFACIAL BONE

12.2.1 STRUCTURE OF CRANIOFACIAL BONE

Eight bones in the cranium along with 14 facial bones make up the craniofacial skeleton. Craniofacial bones are geometrically complex, anisotropic, and hierarchically structured. Bone thickness and density vary with anatomical position (Fig. 12.2A) and between individuals. Elastic modulus distribution within craniofacial bone exhibits high regional and directional variation (Auperrin et al., 2014; Boruah et al., 2017; Motherway, Verschueren, Van der Perre, Vander Sloten, & Gilchrist, 2009; Peterson & Dechow, 2003; Schwartz-Dabney & Dechow, 2003). Cranial bone is generally thicker than midfacial bone, which corresponds to its important function of protecting the brain from external static forces or impacts as well as supporting the circulatory-

**FIGURE 12.2**

Anatomy and bone thickness distribution of craniofacial bone. (A) Thickness of craniofacial bone. (B) Purple arrows show craniofacial buttresses. (C) Blue arrows show vertical pillars in craniofacial bone.

metabolic supply for the brain (Moskalenko et al., 2012). Midfacial bone exhibits large variations in thickness, and some regions (e.g., nasal bone and buccal bone plate of the maxilla) only contain a thin layer of cortical bone with thickness <1 mm (Januário et al., 2011; López-Jarana et al., 2018) often making them susceptible to fracture (Jin et al., 2018; Manson et al., 1999; Zhou, Gong, Hossaini-Zadeh, & Du, 2019). These requirements for thin structures with high mechanical strength ultimately make designing porous scaffold for craniofacial bone repair challenging.

12.2.2 CRANIOFACIAL BONE BIOMECHANICS

Craniofacial bones are made up of a higher percentage of cortical bone and are less enriched in bone marrow compared to long bones. At submillimeter scale, both cortical and trabecular regions comprise of lamellae—thin layers formed by mineralized collagen fibrils and bioapatite. Depending on the function of the bone, some parts are resistant to tension while others are resistant to compression (Portigliatti Barbos, Bianco, Ascenzi, & Boyde, 1984). This variation in mechanical property is realized via the arrangement of lamellae, which remodel in response to physiologic loading. Trabecular bone has higher yield strain than cortical bone (Hayes & Gerhart, 1985). For both cortical and trabecular bone, the ultimate strength under compressive load is higher than under tensile load (Kopperdahl & Keaveny, 1998; Reilly & Burstein, 1975). In considering scaffold design, physiologic forces need to be considered. Two traditional theories have mainly been applied to describe craniofacial bone biomechanics: beam theory and buttress theory. These are important analytical theories in craniofacial bone biomechanics and they help predict the mechanical load transmission in craniofacial bone.

Beam theory is used to study static loadings on skull. According to beam theory, three kinds of forces impact craniofacial bones: forces generated by muscle, occlusal forces, and forces acting on the temporomandibular joint (TMJ; the complex joint contact between the mandibular condyle and the glenoid fossa of temporal bone). When the mandible is at rest, the sum of force components and also the sum of moments of the forces in the sagittal plane are equal to zero. The analytical

solutions for strain distribution in the craniofacial bones based on this theory have been verified with experimental measurements (Barbenel, 1974; Endo, 1970; Pruim, de Jongh, & ten Bosch, 1980) but beam theory remains limited as it treats the mandible as a nondeformable rigid structure. Experimental (Endo, 1965) and numerical (Endo & Adachi, 1988) studies have found that the actual stress and strain distribution within mandible is complex: compressive, tensile, torsional, and shear forces all exist. Also, beam theory does not consider the material property variation within the mandible. Cortical bone and trabecular bone have very different stiffness and are expected to deform differently under masticatory forces. Finally, assumptions about the masticatory forces (Pruim et al., 1980) might not reflect the physiologically realistic loading condition in the mandible during mastication.

Buttress theory reduces the craniofacial skeleton to a frame containing horizontal buttresses and vertical pillars (Fig. 12.2B and C). Midfacial bone contains four main sinuses, which are hollow cavities in the midface mainly in charge of producing mucus to moisturize the nose. These sinuses contain less bone volume and are reinforced by the buttresses and pillars in the craniofacial bone frame. Compressive masticatory forces are transmitted from the maxilla to the zygoma via vertical pillars and horizontal buttresses. The buttresses in cranial bone are formed by the supraorbital bone margins. There are four main mechanical transduction trajectories in the midface. The buttress theory is important in guiding surgery for facial fractures (Grass & Mackinnon, 1986). However, discrepancies between pillar and buttress distribution in buttress theory and the measured *in vivo* stress and strain distributions in craniofacial bone (Pakdel, Whyne, & Fialkov, 2017) indicate that some pillars and beams may not be main load-bearing or load-transmitting structures. There is also some disagreement between the buttress theory and bone remodeling theory. According to the law of bone remodeling proposed by Wolff (1986), bone adapts its density and morphology to reinforce mechanical strength under loading. However, the pillar and buttress trajectories do not always exhibit more density, thicker structures or higher elastic moduli than neighboring regions (Peterson, Wang, & Dechow, 2006). Also, buttress theory mainly emphasizes axial tensile and compressive forces, and the importance of muscle force acting on craniofacial bone is not adequately addressed. Actually, masticatory forces subject craniofacial bone to bending momentum and shear stress (Ross et al., 2011).

Besides these traditional theories, numerical methods are also applied to investigate biomechanics in craniofacial bone. Finite element analysis (FEA) is a powerful numerical technique to solve space- and time-dependent differential equations that usually cannot be solved analytically (other two commonly used numerical methods are finite difference and finite volume methods but they are not widely used in scaffold design). FEA can generate field outputs such as displacement, velocity, temperature, and mechanical stress and strain. It can predict the stress and strain in the mandible under symmetric (Knoell, 1977) and unilateral static mastication. The development of FEA software has made the method popular in simulating biomechanics and multiphase physics processes for complex geometries and boundary conditions. Geometry and bone mineral density information can be imported simultaneously, making element-wise material property assignment possible. Also, complicated boundary conditions at multiple locations can be added accurately on geometries obtained from clinical computed tomography (CT). With material properties and boundary conditions carefully assigned, FEA results show good agreement with experimental measurements in craniofacial bones (Su, Zhou, Hossaini-Zadeh, & Du, 2021). All these make FEA a powerful and efficient tool in studying the complex biomechanics in craniofacial bone.

12.3 MATERIALS FOR CRANIOFACIAL SCAFFOLD

With the quest to find the “ideal” bone graft, numerous materials have evolved over the past few decades and limitless variations in scaffold type and architecture have been developed. The repair of craniofacial bone defects is complex, and a successful reconstruction involves both reconstructive and esthetic realms. The “ideal” bone substitute for craniofacial regeneration is not only expected to replace the missing section of bone but should also provide an optimum osteoconductive and osteoinductive environment for new bone regeneration (Amini, Laurencin, & Nukavarapu, 2012; Tevlin et al., 2014). The biomaterials for craniofacial bone regeneration can be broadly classified into three categories: Bioceramics, polymers, and metals.

12.3.1 BIOCERAMICS AND BIOACTIVE GLASSES

Bioceramics are inorganic biomaterials that have been a popular choice for craniofacial bone regeneration over the past few decades. They are broadly categorized as (1) bioinert ceramics (alumina and zirconia), (2) bioresorbable ceramics (calcium phosphate ceramics), and (3) bioactive ceramics (bioglass, HA) (Vallet-Regí, 2010). Bioinert ceramics, the ones that cannot form a direct bond with the surrounding host tissue, such as alumina (Al_2O_3) and zirconia (ZrO_2), have been particularly popular in maxillofacial reconstructions and postdental applications due to their biocompatibility, desirable mechanical strength, esthetic purposes, and excellent wear and corrosion resistance. However, because of their inertness, they form fibrous tissue growth at the tissue–implant interface, which ultimately leads to implant failure. Bioresorbable calcium phosphate ceramics such as HA and TCP are the most widely used biomaterials in craniofacial defect repair applications due to their tailored resorbability and compositional similarities to the inorganic mineral component of human bone, 85% of which is composed of carbonated HA. HA exhibits a slow and limited resorption rate which can be controlled by tailoring the porous architecture of the graft. Solid nonresorbable HA is used for inert biocompatible fillers in different craniofacial defects and showed potential in alveolar ridge augmentation. On the other hand, TCP has a comparatively higher resorption rate, and it has been successfully used as a resorbable filler in periodontal and alveolar bony defects allowing sufficient time for the replacement with new bone growth. 3D-printed β -TCP scaffolds were able to restore a critical-sized (12 mm) mandibular defects within 8 weeks in adult rabbits (Lopez et al., 2018). Another study exhibited promising bone regeneration by a 3D-printed β -TCP–PCL (poly(ϵ -caprolactone)) scaffold after 8 weeks of implantation in a rabbit calvarial defect (Pae et al., 2019).

Amorphous bioactive glass ceramics is another appealing candidate for its exceptional ability to form a hydroxy-carbonated apatite layer on the glass surface, which serves as a bonding zone to attach firmly with the host bone tissue (Rahaman et al., 2011). 45S5 glass is the gold standard for bioactive glass and its silica-rich surface layer not only enhances its bioactivity but also promotes angiogenesis. The main drawbacks for bioceramics and glass ceramics are their low fracture toughness and strength and therefore are not suited to be used for load-bearing regions of the craniomaxillofacial skeleton. 3D-printed bioglass scaffolds loaded with BMP-2 gene and mesenchymal stem cells (MSCs) demonstrated improved bone healing in a simulated human alveolar bone defect model of primate rhesus monkeys (Wang, Xu, Chen, & Wang, 2019). Another study reported that

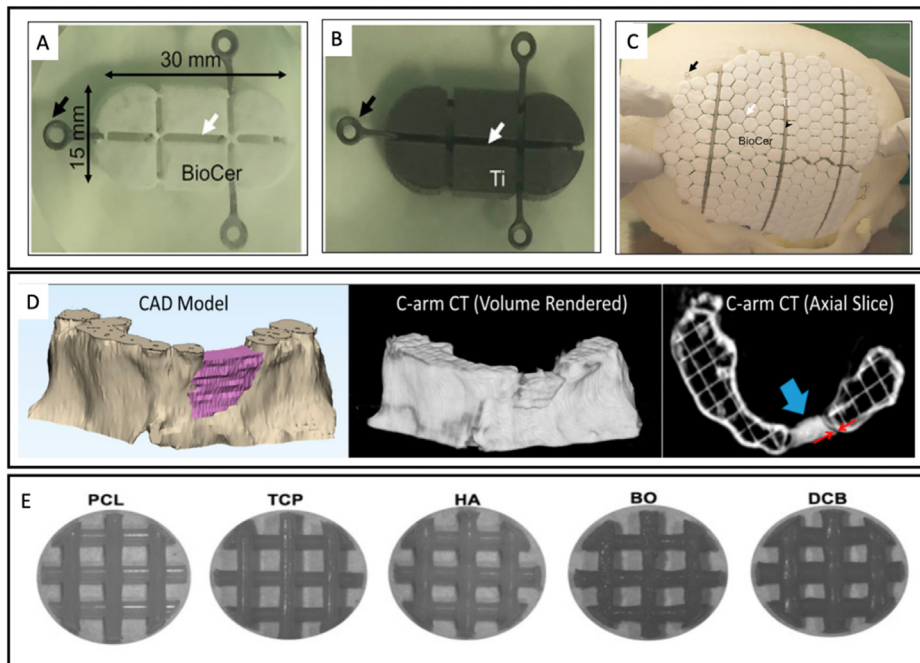
the addition of calcium sulfate hydrate—based bone cement enhanced the poor mechanical properties of 3D-printed mesoporous bioactive glass (MBG) scaffolds while maintaining the optimum biological properties exhibited by MBG (Qi et al., 2017).

In a recent study, a promising synthetic scaffold has been designed by combining 90% HA ceramic and 10% PCL or poly(lactide-*co*-glycolide) (PLGA) polymer that showed exceptional mechanical and biological properties (Jakus et al., 2016). The composite, called “hyperelastic bone,” can be rolled, folded, or cut in complex shapes and independent miniature-scale versions of a human skull, skull cap, mandible, and upper thoracic were successfully created. The scaffolds were able to recover their shape completely upon unloading of compressive force. When these scaffolds were cultured with human mesenchymal stem cells (hMSCs), they showed enhanced cellular proliferation as shown by the live confocal assay and significantly increased osteopontin, osteocalcin, and collagen I expression at 7, 14, and 28 days.

12.3.2 METALS

Metals have been appreciated throughout history because of their excellent mechanical performance as bone grafts. In the early ages, precious metals such as gold and silver were used in cranial reconstruction but later discontinued due to their inherent softness and high price. In the early 20th century, platinum, aluminum, lead, and tantalum were investigated as potential reconstructive materials; however, they caused an increased risk of infections and toxicity (Cho & Gosain, 2004; Mantripragada, Lecka-Czernik, Ebraheim, & Jayasuriya, 2013). Although tantalum is resistant to corrosion, infection, and immunologic reaction, its widespread application is limited due to its high cost. At present, titanium and its alloys are the only metals that have been used for craniofacial reconstruction (Aydin, Kucukyuruk, Abuzayed, Aydin, & Sanus, 2011). Titanium is biocompatible, noncorrosive, noninflammatory, and therefore has lesser chances of graft infection. Besides, it has overall good mechanical strength and malleability, as well as provides excellent cosmesis. Other metals, such as 316 stainless steel and Co—Cr (cobalt—chrome alloy), have been investigated for the development of craniofacial bone plates and screws (Aydin et al., 2011). Yet, these metals are nonbiodegradable and inert, for instance, they do not integrate with host bone or induce new bone formation. Current bone tissue engineering research has a special focus on magnesium (Mg)-based alloy, which is a biodegradable metal and thus can undergo complete degradation and gradual replacement by new bone tissue within 1 year (Pogorielov, Husak, Solodivnik, & Zhdanov, 2017). Mg-based biomaterials also possess lower elastic modulus than other metallic implants and eliminate the stress-shielding phenomenon.

A recent study developed a 3D-printed cranial implant consisting of a titanium-reinforce calcium phosphate bioceramics, referred to as BioCer, to investigate its effect on bone regeneration and osseointegration in large cranial reconstruction (Fig. 12.3A—C). The *in vivo* study carried out in sheep cranial defect showed high percentages of bone area and bone—implant contact by BioCer after 12 months of implantation compared to the control Ti implant. The implant also showed satisfactory defect restoration in a 22-year-old male patient who underwent decompressive hemicraniectomy after an accident. Further investigations after 21 months of cranioplasty with BioCer implant showed prominent integration with the surrounding tissue and bone, with no signs of adverse effect suggesting the implant’s ability to elicit a biological response without the need for adjunct cell therapy or growth factors (Omar et al., 2020).

**FIGURE 12.3**

Craniofacial defect and 3D-printed biomaterials for bone regeneration. (A–C) Design of a 3D-printed cranial implant made of titanium-reinforced calcium phosphate bioceramic, referred to as BioCer. BioCer, composed of calcium phosphate tiles and 3D-printed Ti frame, was implanted in the sheep skull (A), the control implant used in the *in vivo* sheep study was entirely made of 3D-printed grade 23 Ti (B), and BioCer cranial implant used in human skull has a built-in fixation arm to anchor recipient's native skull bone (C) (Omar et al., 2020). (D) CAD model of the cleft-craniofacial defect and cryogel scaffold, volume rendered and axial slice C-arm CT-scan indicating the scaffold placement and gap distance (de la Lastra et al., 2018). (E) Stereoscope images of fused-deposition 3D-printed PCL scaffolds containing TCP, HA, BO, and DCB confirming the presence of mineral stained with alizarin red (Nyberg et al., 2017). PCL, poly(ϵ -caprolactone); TCP, tricalcium phosphate; HA, hydroxyapatite; BO, Bio-Oss; DCB, decellularized bone matrix.

12.3.3 NATURAL AND SYNTHETIC POLYMERS

Polymers are long-chain, organic materials composed of repeating units of monomers. Both naturally derived and synthetic polymers have been valued in craniofacial bone scaffold development due to biocompatibility, controlled degradation, and ease of processing (Thrivikraman et al., 2017). Natural polymers such as collagen, gelatin, alginate, chitosan, and silk are used to make bone tissue engineering scaffolds due to their ability to mimic the structural and chemical components of natural extracellular bone matrix. Collagen is the main structural protein that forms the extracellular matrix of connective tissue in vertebrates and therefore it offers cytocompatibility and stimulates appropriate biological response. A recent study showed, 3D-printed β -TCP/collagen construct

showed improved bone cell proliferation and alkaline phosphatase activity compared to β -TCP scaffold within 3 weeks (Fahimipour et al., 2018). Chitosan and alginates are natural polysaccharides, and their cationic surface charge allows cell attachment and promotes suitable interactions with growth factors that help in tissue regeneration. A study designed 3D-printed chitosan scaffold with graphene oxide (GO) which showed significant enhancement of early and late-stage osteogenesis markers at 8- and 18-week postimplantation in a critical-sized mouse calvarial defect (Hermenean, Ada, Herman, & Balta, 2017). Silk is another natural polymer that supports cellular proliferation and guides *in vitro* and *in vivo* bone formation.

Synthetic polymers such as PCL, polylactic acid (PLA), polyglycolide (PGA), and PLGA ensure better reproducibility and wider control over mechanical properties by tailoring the concentration and degrees of crosslinking or copolymerizing two or more of them. For instance, PLGA, which is a copolymer of PLA (hydrophobic lactide polymer) and PGA (hydrophilic glycolide polymer), offers a varying degree of resorbability, mechanical strength, and osteoconductivity depending on the ratio of lactide to glycolide used for polymerization. A novel ceramic–polymer composite with macroporous PLA with microporous glass–ceramic apatite–wollastonite is designed, which showed higher new bone formation after 12 weeks in a rat calvarial defect model, compared to control PLA (Tcacencu et al., 2018). PCL is another popular, FDA-approved hydrophobic polymer, which is preferred for its high mechanical strength, biocompatibility, and controlled rate of degradation. It undergoes hydrolytic degradation, and its degradation products, CO_2 and H_2O can be easily removed by natural pathways. The main downside of polymeric scaffolds is their poor mechanical property with low compressive strength, which limits their application to nonweight bearing sites of the craniofacial region. Among non-degradable synthetic polymers, polyether ether ketone (PEEK), polymethylmethacrylate (PMMA), and polyethylene (PE) have been investigated to develop various craniofacial implants. Despite possessing low toxicity, biocompatibility, and suitable mechanical performance, these polymers are bioinert and suffer from hydrophobicity, and thus exhibit poor osseointegration with the surrounding host bone tissue. Poly(propylene fumarate) (PPF) is another synthetic polymer with an emerging interest in tissue engineering applications due to its capacity for crosslinking, nontoxic degradation product, and favorable mechanical properties. A recent study investigated the effect of the molecular mass of PPF on degradation and resultant *in vivo* tissue growth and fabricated PPF with two different molecular mass (1000 and 1900 Da) by tailoring the order of polymerization. They have demonstrated that lower molecular mass PPF showed faster bone regeneration in a rat critical-sized calvarial defect at 4 weeks compared to the PPF scaffolds with higher molecular mass; however, the differences in regenerated bone volume were less pronounced at 12 weeks (Nettleton et al., 2019).

12.3.4 HYDROGELS

Hydrogels are formed by a three-dimensional network of cross-linked, hydrophilic polymers that are capable of absorbing large volume of aqueous solution. The physical properties of hydrogels, including porosity, the amount of water absorbed or swelling behavior, as well as, mechanical performance such as stiffness and compressive strength can be altered by tailoring the crosslinking density. The physical properties also influence the viability of cells which can be encapsulated during the hydrogel formation. In general, hydrogels exhibit poor mechanical properties which limit

their application to the low load-bearing areas such as the subcutaneous region in the cranium where stiffness is less important. A 3D-printed cryogel–hydrogel scaffold fabricated was fabricated to create a patient-specific graft to treat cleft-craniofacial defect in children. The computer-aided design (CAD) model of the defect site and the patient-specific scaffold was fitted and scanned by C-arm CT to determine the average maximum gap distance (de la Lastra et al., 2018; Fig. 12.3D). Poly(ethylene glycol) (PEG) is a highly hydrophilic, biocompatible polymer that is most commonly used for synthesizing hydrogels due to its efficient cell encapsulating ability. Hydrogels sensitive to external stimuli including pH, temperature, solvent, light, and pressure electric fields are called “smart” hydrogels. These stimuli-responsive or “smart” hydrogels have received widespread attention, which led the researchers to study and develop a variety of temperature- and pH-sensitive polymers. Among them, poly(*N*-isopropyl acrylamide) (PNIPAM) is a temperature-responsive “smart” polymer that undergoes a phase transition from a swollen, hydrated state to a shrunken, dehydrated state by releasing the absorbed water above 32°C (90°F), which is close to the human body temperature.

12.3.5 DEMINERALIZED AND DECELLULARIZED BONE MATRIX

As an alternative to synthetic scaffolds, natural bone substitutes known as demineralized bone matrices (DBM) are widely used for craniofacial bone regeneration. DBM is typically obtained by removing the mineral component of bone by mild acid extraction, which leaves a composite of non-collagenous proteins, growth factors, and collagen. DBM, as the name suggests, is devoid of the mineralized phase of the bone and thus does not provide structural integrity. However, it is osteoconductive, osteoinductive, as well as provides appropriate stiffness for large craniofacial vault reconstruction. Decellularized bone tissue, on the other hand, is another popular natural alternative, which is formed by removing the cellular components from the native bone ECM (Amini & Lari, 2021; Rindone, Nyberg, & Grayson, 2018). Decellularized ECM is known for its suitable mechanical and structural properties, resemblance to native bone ECM, and lower risk of immune reaction (Chen & Lv, 2018). A recent study has compared 3D-printed PCL scaffold with different additives such as TCP, HA, “Bio-Oss,” and decellularized bone matrix for their printability, mechanical strength, osteoinductivity, calcium content, and osteogenic gene expression. PCL-decellularized bone showed higher collagen 1 and osteocalcin expression as well as significantly higher calcium content compared to other compositions suggesting their potential in bone healing applications over PCL–HA, PCL–TCP, or PCL–Bio-Oss blends (Nyberg, Rindone, Dorafshar, & Grayson, 2017; Fig. 12.3E). Decellularized ECM has been used to mimic the ECM microenvironment and further enhance bone regenerative capabilities of a 3D-printed ceramic–polymer composite in an *in vivo* rat calvaria model. Demineralized and decellularized ECM have been employed with 3D-printed PCL– β -TCP scaffold that exhibited significantly higher new bone volume and area after 4 weeks of surgery in an 8 mm diameter rat calvarial defect model (Bin Bae et al., 2018). Decellularized ECM from an autogenous skull bone flap has been used in 3D printing which facilitated mineralization, promoted vascularized bone formation, and successfully restored a critical-sized skull defect after 3 months of implantation (Chen et al., 2019). A porcine-derived ECM has been used along with PCL/ β -TCP which showed excellent *in vivo* bone regeneration in a rat calvarial defect model (Kim et al., 2018).

12.4 3D-PRINTING TECHNIQUES FOR CRANIOFACIAL SCAFFOLD

In the past three decades, additive manufacturing or three-dimensional printing (3DP) revolutionized the concept of manufacturing bone scaffolds. 3DP spans a variety of techniques; however, the main concept of 3DP remains the same: it involves layer-by-layer processing from CAD models to manufacture customized or complex structures without the need for part-specific molds or machining. 3DP for biomaterials can be broadly categorized into four main techniques, namely photopolymerization, extrusion-based 3D printing, laser-assisted printing, and inkjet printing ([Rider et al., 2018](#)).

12.4.1 PHOTOPOLYMERIZATION

Stereolithography (SLA) is the first commercialized 3D-printing process that is based on the principle of photopolymerization. It involves the use of photocurable, liquid monomers/oligomers that are cured and hardened on a build bed by ultraviolet light. SLA offers advantages in tissue engineering by constructing high-resolution complex structures ($<100\ \mu\text{m}$); however, the process is not sufficiently versatile to be used for a range of materials and multimaterial printing ([Bagheri & Jin, 2019](#)).

12.4.2 EXTRUSION-BASED PRINTING

Extrusion-based printing, also referred to as fused deposition modeling (FDM), is one of the most popular 3D-printing techniques in the tissue engineering field because of its simplicity, ease of use, fast processing speed, and low cost. In FDM, thermoplastic materials in the form of a continuous filament are fed into a heated extruder, where it is softened and deposited through a nozzle by mechanical or pneumatic pressure. The printhead moves in a dictated x – y pattern allowing it to form a complex 3D object. Compared to other 3DP techniques, FDM offers lower resolution ($>100\ \mu\text{m}$) and requires high-viscosity inks. A large variety of materials can be printed with this technique. However, in order to print ceramic or metal parts, the powders need to be premixed with polymers and extruded in a feedstock filament form prior to printing. Extrusion-based 3D printing also allows multimaterial printing and therefore scaffolds with compositional gradients can be fabricated by this technique. Various polymer–ceramic composites such as PPF with osteoconductive HA nanoparticles ([Trachtenberg, Placone, Smith, Fisher, & Mikos, 2017](#)), PEEK/HA biocomposites ([Vaezi et al., 2016](#)), PCL/ β -TCP composite membrane, PCL/PLGA/ β -TCP scaffold, and poly(ester urea) blended with HA nanocrystals ([Yu, Xu, Li, Seifert, & Becker, 2017](#)) have been fabricated via extrusion-based 3D printers for craniofacial bone regeneration.

12.4.3 LASER-ASSISTED 3D PRINTING

Laser-assisted 3D printing, also known as powder bed fusion or selective laser sintering (SLS), involves a focused laser beam or electron beam, which selectively “fuses” polymer or ceramic powder particles in the build bed. SLS does not require any solvent and thus eliminates the risk of shrinkage during postprocessing. Another advantage of this process is the use of powder bed as

support material, which acts as a temporary support structure for the complex 3D parts. Recently, a study showed successful fabrication of a biphasic calcium phosphate-based scaffold by indirect SLS printing method, where the epoxy resin was used during printing, later decomposed, and then eliminated from the final part. The resultant microporous scaffold showed enhanced bone regeneration and vascularization in a rabbit critical-sized cranial model (Zeng et al., 2020). 3D-printed polylactic acid/nanohydroxyapatite (PLA/nHA) composite scaffolds were fabricated by FDM, which showed enhanced osteogenesis and osteoconductivity in an *in vivo* rabbit critical-sized defect model (Chen et al., 2019). SLS is primarily used for polymers and ceramic materials printing; however, to enable printing of metallic parts, the laser beam is employed to “melt” the metal powder and the technique is referred to as “selective laser melting”.

12.4.4 BINDER JETTING

Inkjet printing, or in other terms, binder jetting is another promising 3D-printing technique widely used in the tissue engineering field to fabricate high-resolution ($<100\ \mu\text{m}$), customized parts with complex geometries. In this technique, droplets of low-viscosity binder solution are selectively deposited from an inkjet head, which moves in the x – y direction as directed by the CAD file. The binder is selectively deposited on the bulk material at powder bed, and it helps to “glue” the particles together. The binder-jetting process involves extensive postprocessing stages where the binder needs to be “cured” at an optimum temperature to provide green strength to the printed part. Although binder composition and droplet size need to be optimized to ensure good quality parts, binder jetting is of particular interest in biomedical application due to its room temperature processing and versatility in printing ceramic, polymer, and metal parts.

12.5 ENHANCING THE REGENERATIVE CAPABILITY OF BIOMATERIALS IN CRANIOFACIAL BONE REGENERATION

12.5.1 EFFECT OF SCAFFOLD GEOMETRY

3DP enables the formation of complex hierarchical structures that closely mimic the surface topography and nano- and microscale environment of native bone tissue. For instance, one study developed a 3D-printed PCL scaffold with micropatterned PCL thin film consisting of grooved pillars to replicate the alveolar bone-periodontal ligament region. The result showed 3D-printed micropatterned scaffolds fabricated by standard photolithography which promoted cellular alignment and collagenous tissue orientation in an *in vivo* murine periodontal osseous defect model (Pilipchuk et al., 2016). Another study demonstrated that an extrusion-based 3DP PCL/ β -TCP scaffolds with heterogeneous pore sizes and additional wing-like structures resulted in higher bone formation in a large critical-sized defect in canine mandibles (Lee, Choi, Shim, & Nam, 2020). The effect of different geometric patterns on the biological performance of 3D-printed scaffolds has also been investigated, where small square pores were fabricated via a novel table-top stereolithography 3D printer. In this study, a PEG diacrylate bioink containing RGDS (arginine–glycine–aspartic acid–serene) peptide was printed using a stereolithography-based 3D bioprinter that promoted MSC proliferation and osteogenic

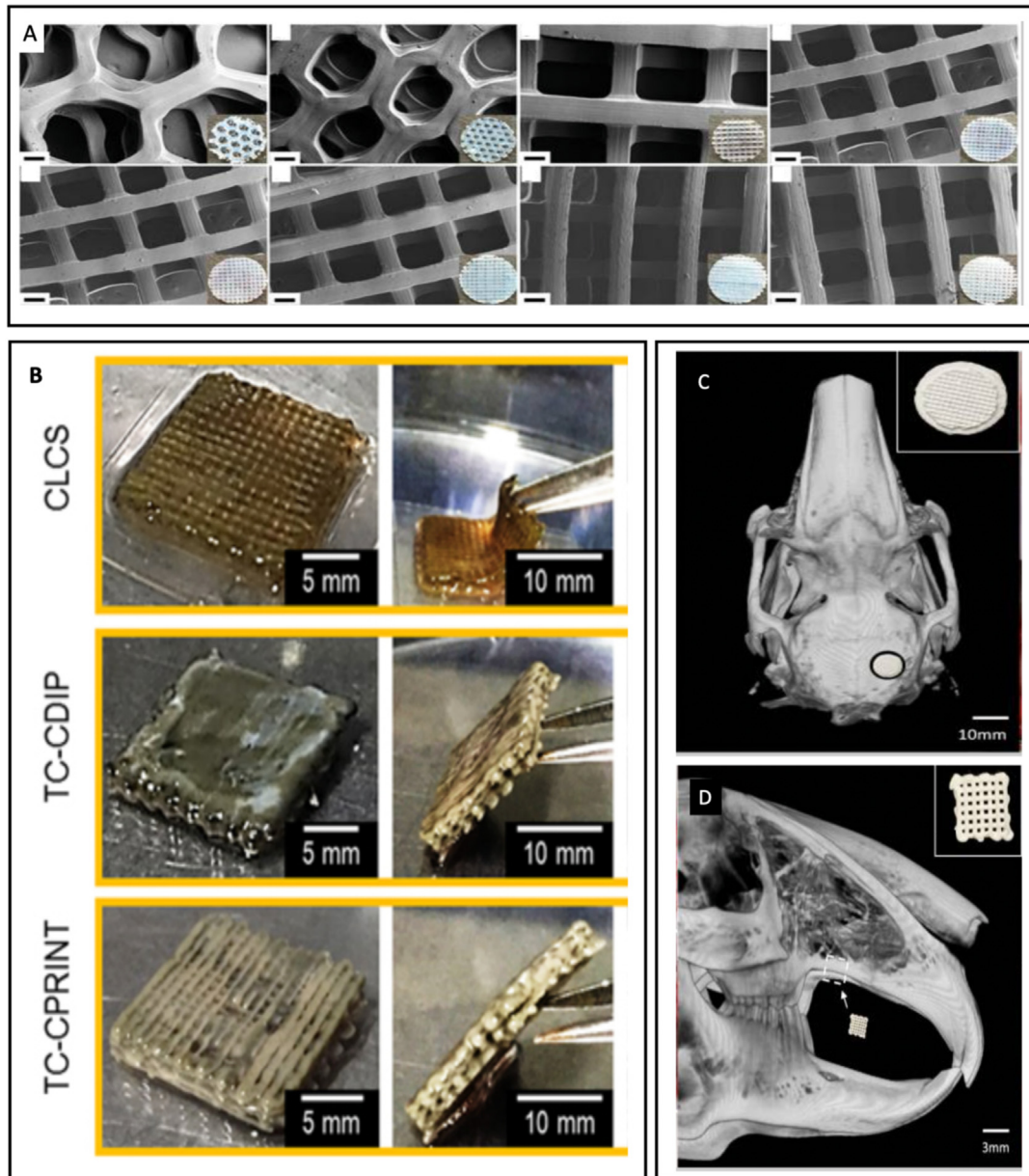
differentiation (Fig. 12.4A; Zhou et al., 2016). The optimal scaffold pore size for osteogenesis is another area of investigations, which has shown inconsistent outcomes over the years: nanocomposite polydopamine-laced hydroxyapatite collagen calcium silicate (HCCS-PDA) scaffolds with 500 μm pores showed greater bone regeneration in a rat critical-sized calvarial defect compared to a 250 μm pore size scaffold (Lee et al., 2019). However, in another study, 3D-printed β -TCP scaffold with 100 μm pore size showed the highest percentage of new bone ingrowth compared to 250 and 400 μm pore size scaffold in a rat critical-sized calvarial model (Diao et al., 2018). In terms of porosity, 30% porosity in a 3D-printed PCL membrane showed higher osteogenic differentiation compared to the membranes with 50% and 70% porosity, although cell proliferation was unaltered (Shim et al., 2018).

12.5.2 EFFECT OF MESENCHYMAL STEM CELLS, PROGENITOR CELLS DELIVERY

Bioprinting, the newest addition in the 3D-printing technology allows precise printing of cells as directed by a computer-generated pattern (Datta, Ozbolat, Ayan, Dhawan, & Ozbolat, 2017; Visscher et al., 2016). 3D-printed cell-laden scaffolds are being widely investigated in bone tissue engineering since they transfer bioactive cues to guide bone regeneration and thus overcome the shortcomings of conventional 3D-printed scaffolds (Grayson et al., 2015). A 3D-printed α -TCP/collagen scaffold with cell-laden collagen bioink is designed which showed significantly higher mechanical properties and biological activities compared to pure cell-laden collagen scaffolds (Fig. 12.4B; Kim, Yun, & Kim, 2017). A similar ceramic-hydrogel-based bioink, 3D porous β -TCP-collagen with preosteoblasts (MC3T3-E1) and human adipose stem cells were fabricated that showed significant osteogenic activities and satisfactory mechanical strength (Kim & Kim, 2020). Another promising bioink was developed by incorporating 3% alginate/GO composites with MSCs, which showed good printability, structural stability, and osteogenic induction (Choe, Oh, Seok, Park, & Lee, 2019). In a recent study, titanium implants with designed features were fabricated by powder bed fusion method and the implants were coated with stromal cell-derived factor-1 followed by the infiltration of stem cell. The *in vivo* study showed that bone marrow stem cell recruitment into titanium implants improved osseointegration 12 weeks after surgery in a rabbit cranial defect (Bollman et al., 2020).

12.5.3 EFFECT OF DOPANTS OR COATING

Recent studies showed that the presence of 10% zinc silicate in an HA/collagen scaffold significantly enhanced *in vivo* bone regeneration in a rat critical-sized cranial defect model (Song et al., 2020). The essential trace element, zinc, is crucial for bone formation and mineralization whereas silicon stimulated angiogenesis and osteogenic differentiation. The scaffold promoted osteogenic differentiation of monocytes into TRAP⁺ cells, which expressed higher levels of cytokines to regulate bone marrow stromal cells and endothelial cells to the defect site and subsequently enhanced osteogenesis and angiogenesis. Strontium, another trace element, stimulated bone formation, osteogenic differentiation, and inhibited osteoclastic bone resorption. 3D-printed Sr-substitutes in HA scaffolds showed higher osteogenesis within 12 weeks of surgery in critical-sized rabbit calvarial defects with a diameter of 15 mm (Luo, Chen, Shi, & Ma, 2018). Mg is one of the most abundant ions found in our body and it has been reported that it also has an effect on bone metabolism as

**FIGURE 12.4**

Enhancing the regenerative capability of biomaterials in craniofacial bone regeneration. (A) 3D bio-printed scaffold with varying geometry (large hexagonal, small hexagonal, large square, and small square) and

(Continued)

well as new bone formation. A 3D-printed Mg-doped β -TCP scaffold showed higher osteogenesis and angiogenesis biomarkers *in vitro* (Gu et al., 2019). Ceramics and polymeric biomaterials have been widely used as coatings to improve *in vivo* tissue–implant contact. A recent study demonstrated polydopamine coating on 3D-printed PLGA/ β -TCP scaffold successfully repaired mouse skull defects and exhibited better osteogenic potential compared to the uncoated control (Xu et al., 2019).

12.5.4 EFFECT OF OXYGEN AND GROWTH FACTORS DELIVERY

A prominent application of 3D-printed scaffolds is their ability to deliver therapeutics at the desired tissue site. Over the years, 3D-printed grafts have been utilized to successfully deliver various natural or synthetic drugs, growth factors, stem cells, or differentiated matured cells to promote bone regeneration (Perez et al., 2018; Tollemar et al., 2016). Several studies in the recent past have investigated biomaterials with the potential for oxygen delivery, which have shown promising results in regulating wound microenvironment and promoting bone regeneration (Farris, Cook, & Grayson, 2019). Among all growth factors, bone morphogenic proteins (BMPs), especially BMP2 and BMP7, are most widely researched for bone regeneration due to their clinical approval by the FDA. However, the interest in BMPs has considerably lowered due to their high cost, short half-life. Recently bone-forming peptide-1, a short peptide derived from BMP7, is developed, which showed satisfactory osteo-promoting ability in an 8-week *in vivo* rabbit calvarial model (Lee et al., 2019). Similarly, a BMP-2 like osteogenic peptide has been increasingly used as a potential alternative to BMP-2. An *in vivo* study showed that PLGA/ β -TCP/GO scaffold promoted bone volume in the critical-sized defects 4 and 12 weeks postimplantation in critical-sized rat cranial defect (Zhang et al., 2019). Another BMP-2-derived oligopeptide, SSVPT (Ser-Ser-Val-Pro-Thr) was conjugated with a dopamine coating and loaded onto a 3D-printed PLA scaffold to enhance osteogenesis in a rat cranial defect model.

12.5.5 EFFECT OF DRUG DELIVERY

In the past few decades, numerous natural and synthetic medicines have been incorporated in tissue engineering scaffolds to enhance bone regeneration at the defect site (Bose & Sarkar, 2020). The major advantage of localized drug delivery is the ability to provide high concentrations at the localized area to eliminate the need for large oral dosages and reduce the risk of systemic toxicity. The localized drug delivery is also advantageous to treat bone pathologies or complications such as

different biomaterials—bioink combination which includes nano hydroxyapatite (nHA), photocurable polyethylene (glycol) diacrylate bioink (PEGDA), arginine—glycine—aspartic acid—serine (RGDS) peptide, common integrin binding sequence (Zhou et al., 2016). (B) 3D-printed cell-laden collagen scaffold (CLCS), TC-CDIP scaffold fabricated by dipping the α -TCP/collagen mesh structure into the cell/collagen mixture solution, TC-CPRINT fabricated using the two-step process: printing of α -TCP/collagen struts and then printing cell-laden collagen onto the α -TCP/collagen struts (Kim et al., 2017). Schematic representation of a unilateral calvarial (C) and alveolar (D) defect and implanted 3D-printed β -TCP scaffold for local delivery of dipyridamole (Wang et al., 2019).

infection, inflammation, or bone tumor (Sarkar & Bose, 2020). A study showed, controlled release of dexamethasone, an antiinflammatory corticosteroid from a 3D-printed biodegradable polymer scaffold, successfully suppressed lipopolysaccharide-induced interleukin-6 and nitric oxide synthase secretion by M1 macrophages murine RAW 264.7 cells. The *in vivo* study indicated that scaffolds containing dexamethasone promoted osteoinduction and osteogenic responses at rat calvarial defects (Li et al., 2018). Dipyridamole, a vasodilator, is known for its osteoinductive potential and recently has been utilized with a 3D-printed 100% β -TCP scaffold to treat calvarial and alveolar defects in a pediatric rabbit model. The result showed dipyridamole scaffolds enhanced osteogenic regeneration in the defect area comparable to autogenous bone graft after 24 weeks of implantation (Fig. 12.4C and D; Wang et al., 2019)

12.5.6 EFFECT OF GENE DELIVERY

One of the most recent advancements in scaffold-based therapeutics delivery has been achieved through gene delivery, especially with the use of microRNAs (miRNAs), which have gained significant attention in craniofacial bone regeneration (Curtin, Castaño, & O'Brien, 2018). miRNA is a single-stranded, noncoding RNA molecule that offers a number of therapeutic potentials through mRNA activation and gene expression at the posttranslational stage. Several miRNAs namely miR-31, miR-26a, miR-148b, miR-20a, and miR-135 have been identified that induce osteogenesis in osteoprogenitor cells and stem cells with the help of viral or nonviral vectors, which allow miRNA to pass through the cell membrane and protect them from *in vivo* degradation. In a recent work, 3D-printed hybrid PCL/PLGA/HAp scaffolds infiltrated with collagen have been developed, which are further employed for the delivery of miR-148b transfected rBMSCs. The scaffolds enriched with the miR-148b gene promote osteogenic differentiation and improved bone regeneration in a critical-sized rat calvarial bone defects at 8 weeks postimplantation (Moncal et al., 2019).

12.6 CASE STUDIES: APPLICATION OF POROUS SCAFFOLD DESIGN FOR CLINICAL APPLICATIONS

Load-bearing capacity is a crucial element of craniofacial bone scaffold design. The anticipated mechanical loading needs to be carefully evaluated. While porous scaffolds provide high surface area for cell attachment, larger space for diffusion of nutrients and multiple growth factors, the mechanical strength generally decreases with increasing porosity. Therefore porosity and mechanical strength need to be balanced for mechanical performance and scaffold permeability. FEA tools can optimize material distribution (Sutradhar, Paulino, Miller, & Nguyen, 2010). Scaffolds with anisotropic structures similar to natural trabecular bone have also been developed (Ghouse et al., 2019). Recent developments in 3D printing make nonhomogenous porosity high-resolution scaffold printing possible and bring more flexibility to FEA-assisted scaffold design. The mechanical requirements vary for three broad regions of the craniofacial skeleton—the skull, midface, and jaw.

12.6.1 CASE STUDY 1: CRANIAL DEFECT

A patient with two cranial defects (one defect on each side of skull) was treated with polymer scaffolds and the scaffolds were fixed with three metal pins and wires (Fig. 12.5A). The pins are slightly extruded virtually for illustration. Expected physiologically realistic mechanical forces acting on scaffold could be static compression of skull weight (approximately 10 lb) on scaffold during sleeping or cyclic masticatory forces transmitted to skull during chewing. While designing cranial scaffold, the strength of bond between metal pin, cranial bone, and scaffold should be able to withstand these static mechanical forces in long term.

12.6.2 CASE STUDY 2: MAXILLARY DEFECT

Along with the mandible, the maxilla is the main load-bearing structures during mastication. Safe masticatory forces should be carefully estimated during scaffold design to avoid fracture and, at the same time, to maintain normal function of the maxilla after transplantation. Furthermore, under the conditions of large defects in the maxilla and mandible, the scaffold needs to provide anchorage for teeth as well. With the same material, the mechanical strength of the scaffold will be compromised after leaving out space for teeth substitutes. We analyzed a patient with an anterior maxilla defect in whom a scaffold was designed and fitted into the defect region virtually (Fig. 12.5B). While masticatory forces magnitude vary cyclically during chewing—the mean full clench force is about 365 N on molar, and about 60 N on premolar and incisor (Kumagai et al., 1999)—the maximum chewing force can go beyond 400 N (Hidaka, Iwasaki, Saito, & Morimoto, 1999; Kumagai et al., 1999). Around 10%–20% of the total occlusal force is expected to be transmitted to the defect region (Kumagai et al., 1999). The local biting pressure depends on the contact area between the teeth and the scaffold and is expected to be quite different with or without dental tissue in maxilla defect. Mechanical stimulus affects bone formation and bone resorption and hence affects bone morphology. It may be important to maintain moderate mechanical stimulus around the defect region during healing to prevent progressive and irreversible alveolar bone resorption.

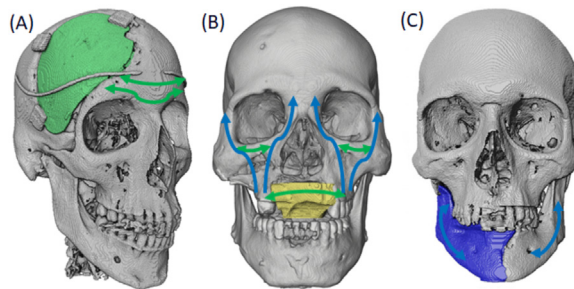


FIGURE 12.5

Patients with craniofacial bone defect. Reconstructed computed tomography (CT) scans of patients with: (A) a cranial defect treated with nonbiodegradable scaffold (green); (B) a maxillary defect with predesigned maxilla scaffold (yellow) virtually implanted; and (C) mandibular defect with scaffold (blue) designed based on geometry of the contralateral, intact half mandible.

12.6.3 CASE STUDY 3: MANDIBULAR DEFECT

We examined a patient with a mandibular defect in which almost half of the mandible needed to be replaced. A mandibular scaffold including the TMJ was designed on the basis of the geometry of the undamaged half (Fig. 12.5C). The mandibular condyle contains less bone compared to other regions in mandible, and the mechanical strength of condyle needs to be specially considered during surgery planning. Specifically, within biodegradable scaffold, bone formation and scaffold degradation may happen simultaneously. Experimental data on condyle movement during chewing have been reported (Miyowaki, Tanimoto, Kawakami, Sugimura, & Takano-Yamamoto, 2001; Naeije & Hofman, 2003), but mechanical load distribution in condyle is very limited due to technical difficulty. Numerical methods have been applied to identify main load-bearing region in condyle (Gregolin, Zavaglia, Tokimatsu, & Pereira, 2017). Regions experiencing higher strain might require extra material strength compared to other parts of the scaffold, possibly by altering the local material composition or by decreasing the local porosity.

The design principles guiding scaffold structure in Case 1 (non-load bearing) differ from the load-bearing situations in Cases 2 and 3. The cranial scaffold primarily protects the brain tissue under static loads with amplitudes similar to the skull weight. Hence, the mechanical strength of scaffold is not a major limitation. As such, larger surface areas, greater permeability, better biocompatibility, and degradability are important for optimal performance. However, load-bearing scaffolds need to maintain mechanical strength during healing. The design optimization principle in Cases 2 and 3 would maximize porosity and permeability while ensuring that mechanical strength requirements are satisfied. Although general ranges of masticatory forces might be gleaned from the literature, some patient-specific factors should be considered. For example, unilateral chewing will add either excessive or inadequate mechanical stimuli and some biting and teeth grinding provide shear forces to the scaffold.

12.7 Conclusion

3D-printing technologies hold tremendous promise for craniofacial bone reconstruction. The scaffolds serve as substrates for attachment, survival, and differentiation of transplanted cells as well as conduits for vascular infiltration and new bone deposition. For defects in load-bearing regions, the scaffold provides temporary mechanical support until adequate bone healing is achieved. The ability to tailor the scaffolds' bioactive and mechanical properties by changing the material, structure, fabrication technique, and biochemical agents makes 3D printing a versatile technique for engineering scaffolds optimized for restoring facial features and function.

References

- Allareddy, V., Allareddy, V., & Nalliah, R. P. (2011). Epidemiology of facial fracture injuries. *Journal of Oral and Maxillofacial Surgery: Official Journal of the American Association of Oral and Maxillofacial Surgeons*, 69(10), 2613–2618. Available from <https://doi.org/10.1016/j.joms.2011.020.057>.

- Allsopp, B. J., Hunter-Smith, D. J., & Rozen, W. M. (2016). Vascularized versus nonvascularized bone grafts: what is the evidence? *Clinical Orthopaedics and Related Research*, 474(5), 1319–1327.
- Amini, A. R., Laurencin, C. T., & Nukavarapu, S. P. (2012). Bone tissue engineering: Recent advances and challenges. *Critical Reviews in Biomedical Engineering*, 40(5), 363–408. Available from <https://doi.org/10.1615/CritRevBiomedEng.v40.i5.10>.
- Amini, Z., & Lari, R. (2021). A systematic review of decellularized allograft and xenograft–derived scaffolds in bone tissue regeneration. *Tissue and Cell*, 69. Available from <https://doi.org/10.1016/j.tice.2021.101494>.
- Auperrin, A., Delille, R., Lesueur, D., Bruyère, K., Masson, C., & Drazétic, P. (2014). Geometrical and material parameters to assess the macroscopic mechanical behaviour of fresh cranial bone samples. *Journal of Biomechanics*. Available from <https://doi.org/10.1016/j.jbiomech.2013.100.060>.
- Aydin, S., Kucukyuruk, B., Abuzayed, B., Aydin, S., & Sanus, G. Z. (2011). Cranioplasty: Review of materials and techniques. *Journal of Neurosciences in Rural Practice*, 2(2). Available from <https://doi.org/10.4103/0976-3147.83584>.
- Bagheri, A., & Jin, J. (2019). Photopolymerization in 3D printing. *ACS Applied Polymer Materials*, 1(4). Available from <https://doi.org/10.1021/acsapm.8b00165>.
- Barbenel, J. C. (1974). The mechanics of the temporomandibular joint—A theoretical and electromyographical study. *Journal of Oral Rehabilitation*. Available from <https://doi.org/10.1111/j.1365-2842.1974.tb01262.x>.
- Bin Bae, E., Park, K. H., Shim, J. H., Chung, H. Y., Choi, J. W., Lee, J. J., et al. (2018). Efficacy of rhBMP-2 loaded PCL/ β -TCP/bdECM scaffold fabricated by 3D printing technology on bone regeneration. *BioMed Research International*, 2018. Available from <https://doi.org/10.1155/2018/2876135>.
- Bollman, M., Malbrue, R., Li, C., Yao, H., Guo, S., & Yao, S. (2020). Improvement of osseointegration by recruiting stem cells to titanium implants fabricated with 3D printing. *Annals of the New York Academy of Sciences*, 1463(1). Available from <https://doi.org/10.1111/nyas.14251>.
- Boruah, S., Subit, D. L., Paskoff, G. R., Shender, B. S., Crandall, J. R., & Salzar, R. S. (2017). Influence of bone microstructure on the mechanical properties of skull cortical bone — A combined experimental and computational approach. *Journal of the Mechanical Behavior of Biomedical Materials*. Available from <https://doi.org/10.1016/j.jmbbm.2016.090.041>.
- Bose, S., & Sarkar, N. (2020). Natural medicinal compounds in bone tissue engineering. *Trends in Biotechnology*, 38(4), 404–417. Available from <https://doi.org/10.1016/j.tibtech.2019.110.005>.
- Bostrom, M. P. G., & Seigerman, D. A. (2005). The clinical use of allografts, demineralized bone matrices, synthetic bone graft substitutes and osteoinductive growth factors: A survey study. *HSS Journal: The Musculoskeletal Journal of Hospital for Special Surgery*, 1(1). Available from <https://doi.org/10.1007/s11420-005-0111-5>.
- Chen, G., & Lv, Y. (2018). Decellularized bone matrix scaffold for bone regeneration. *Methods in Molecular Biology*, 1577, 239–254.
- Chen, H., Zhang, J., Li, X., Liu, L., Zhang, X., Ren, D., & Xu, T. (2019). Multi-level customized 3D printing for autogenous implants in skull tissue engineering. *Biofabrication*, 11(4). Available from <https://doi.org/10.1088/1758-5090/ab1400>.
- Chen, X., Gao, C., Jiang, J., Wu, Y., Zhu, P., & Chen, G. (2019). 3D printed porous PLA/nHA composite scaffolds with enhanced osteogenesis and osteoconductivity *in vivo* for bone regeneration. *Biomedical Materials (Bristol, England)*, 14(6). Available from <https://doi.org/10.1088/1748-605X/ab388d>.
- Cho, Y. R., & Gosain, A. K. (2004). Biomaterials in craniofacial reconstruction. *Clinics in Plastic Surgery*, 31(3), 377–385. Available from <https://doi.org/10.1016/j.cps.2004.030.001>.
- Choe, G., Oh, S., Seok, J. M., Park, S. A., & Lee, J. Y. (2019). Graphene oxide/alginate composites as novel bioinks for three-dimensional mesenchymal stem cell printing and bone regeneration applications. *Nanoscale*, 11(48). Available from <https://doi.org/10.1039/c9nr07643c>.
- Curtin, C. M., Castaño, I. M., & O'Brien, F. J. (2018). Scaffold-based microRNA therapies in regenerative medicine and cancer. *Advanced Healthcare Materials*, 7(1). Available from <https://doi.org/10.1002/adhm.201700695>.

- Datta, P., Ozbolat, V., Ayan, B., Dhawan, A., & Ozbolat, I. T. (2017). Bone tissue bioprinting for craniofacial reconstruction. *Biotechnology and Bioengineering*, 114(11). Available from <https://doi.org/10.1002/bit.26349>.
- De Boer, H. H. (1988). The history of bone grafts. *Clinical Orthopaedics and Related Research*. Available from <https://doi.org/10.1097/00003086-198801000-00037>.
- de la Lastra, A. A., Hixon, K. R., Aryan, L., Banks, A. N., Lin, A. Y., Hall, A. F., & Sell, S. A. (2018). Tissue engineering scaffolds fabricated in dissolvable 3D-printed molds for patient-specific craniofacial bone regeneration. *Journal of Functional Biomaterials*, 9(3). Available from <https://doi.org/10.3390/jfb9030046>.
- Diao, J., Yang, J. O., Deng, T., Liu, X., Feng, Y., & Zhao, N. (2018). 3D-plotted beta-tricalcium phosphate scaffolds with smaller pore sizes improve *in vivo* bone regeneration and biomechanical properties in a critical-sized calvarial defect rat model. *Advanced Healthcare Materials*, 7(17). Available from <https://doi.org/10.1002/adhm.201800441>.
- Elsalanty, M. E., & Genecov, D. G. (2009). Bone grafts in craniofacial surgery. *Craniofacial Trauma & Reconstruction*, 2(3–4), 125–134.
- Endo, B. (1965). Distribution of stress and strain produced in the human facial skeleton by the masticatory force. *Journal of the Anthropological Society of Nippon*. Available from <https://doi.org/10.1537/ase1911.730.123>.
- Endo, B. (1970). Analysis of stresses around the orbit due to masseter and temporalis muscles respectively. *Journal of the Anthropological Society of Nippon*. Available from <https://doi.org/10.1537/ase1911.780.251>.
- Endo, B., & Adachi, K. (1988). Biomechanical simulation study on the forms of the frontal bone and facial bones of the recent human facial skeleton by using a two-dimensional frame model with stepwise variable cross-section members. *Okajimas Folia Anatomica Japonica*. Available from https://doi.org/10.2535/ofaj1936.64.6_335.
- Fahimipour, F., Dashtimoghdam, E., Rasoulboroujeni, M., Yazdimamaghani, M., Khoshroo, K., Yadegari, A., ... Tay, L. (2018). Collagenous matrix supported by a 3D-printed scaffold for osteogenic differentiation of dental pulp cells. *Dental Materials: Official Publication of the Academy of Dental Materials*, 34(2). Available from <https://doi.org/10.1016/j.dental.2017.100.001>.
- Farris, A. L., Cook, C. A., & Grayson, W. L. (2019). Mathematical modeling of oxygen release from hyperbarically loaded polymers. *Biotechnology Progress*, 35(2). Available from <https://doi.org/10.1002/btpr.2751>.
- Ghouse, S., Reznikov, N., Boughton, O. R., Babu, S., Geoffrey Ng, K. C., Blunn, G., Jeffers, J. R. T. (2019). The design and *in vivo* testing of a locally stiffness-matched porous scaffold. *Applied Materials Today*, 15, 377–388. Available from <https://doi.org/10.1016/j.apmt.2019.020.017>.
- Grass, J. S., & Mackinnon, S. E. (1986). Complex maxillary fractures: Role of buttress reconstruction and immediate bone grafts. *Plastic and Reconstructive Surgery*. Available from <https://doi.org/10.1097/00006534-198607000-00002>.
- Grayson, W. L., Bunnell, B. A., Martin, E., Frazier, T., Hung, B. P., & Gimble, J. M. (2015). Stromal cells and stem cells in clinical bone regeneration. *Nature Reviews Endocrinology*. Available from <https://doi.org/10.1038/nrendo.20140.234>.
- Gregolin, R. F., Zavaglia, C. A. D. C., Tokimatsu, R. C., & Pereira, J. A. (2017). Biomechanical stress and strain analysis of mandibular human region from computed tomography to custom implant development. *Advances in Materials Science and Engineering*. Available from <https://doi.org/10.1155/2017/7525897>.
- Griffin, K. S., Davis, K. M., McKinley, T. O., Anglen, J. O., Chu, T.-M. G., Boerckel, J. D., & Kacena, M. A. (2015). Evolution of bone grafting: Bone grafts and tissue engineering strategies for vascularized bone regeneration. *Clinical Reviews in Bone and Mineral Metabolism*, 13(4). Available from <https://doi.org/10.1007/s12018-015-9194-9>.
- Gu, Y., Zhang, J., Zhang, X., Liang, G., Xu, T., & Niu, W. (2019). Three-dimensional printed Mg-doped β -TCP bone tissue engineering scaffolds: Effects of magnesium ion concentration on osteogenesis and angiogenesis *in vitro*. *Tissue Engineering and Regenerative Medicine*, 16(4). Available from <https://doi.org/10.1007/s13770-019-00192-0>.

- Hardt, N., & Kuttnerberger, J. (2010). *Craniofacial trauma: Diagnosis and management*. . .
- Hayes, W., & Gerhart, T. (1985). Biomechanics of bone: Applications for assessment of bone strength. *Journal of Bone Mineral Research*, 3, 259–294.
- Hermenean, A., Ada, C., Herman, H., & Balta, C. (2017). Chitosan-graphene oxide 3D scaffolds as promising tools for bone regeneration in critical-size mouse calvarial defects. *Scientific Reports*, 7(1). Available from <https://doi.org/10.1038/s41598-017-16599-5>.
- Hidaka, O., Iwasaki, M., Saito, M., & Morimoto, T. (1999). Influence of clenching intensity on bite force balance, occlusal contact area, and average bite pressure. *Journal of Dental Research*. Available from <https://doi.org/10.1177/00220345990780070801>.
- Jackson, I. T., Helden, G., & Marx, R. (1986). Skull bone grafts in maxillofacial and craniofacial surgery. *Journal of Oral and Maxillofacial Surgery: Official Journal of the American Association of Oral and Maxillofacial Surgeons*, 44(12). Available from [https://doi.org/10.1016/S0278-2391\(86\)80048-9](https://doi.org/10.1016/S0278-2391(86)80048-9).
- Jakus, A. E., Rutz, A. L., Jordan, S. W., Kannan, A., Mitchell, S. M., Yun, C., Shah, R. N. (2016). Hyperelastic ‘bone’: A highly versatile, growth factor–free, osteoregenerative, scalable, and surgically friendly biomaterial. *Science Translational Medicine*, 8(358). Available from <https://doi.org/10.1126/scitranslmed.aaf7704>, pp. 358ra127.
- Januário, A. L., Duarte, W. R., Barriviera, M., Mesti, J. C., Araújo, M. G., & Lindhe, J. (2011). Dimension of the facial bone wall in the anterior maxilla: A cone-beam computed tomography study. *Clinical Oral Implants Research*. Available from <https://doi.org/10.1111/j.1600-0501.2010.02086.x>.
- Jin, K.-S., Lee, H., Sohn, J.-B., Han, Y.-S., Jung, D.-U., Sim, H.-Y., & Kim, H.-S. (2018). Fracture patterns and causes in the craniofacial region: An 8-year review of 2076 patients. *Maxillofacial Plastic and Reconstructive Surgery*. Available from <https://doi.org/10.1186/s40902-018-0168-y>.
- Kim, J. Y., Ahn, G., Kim, C., Lee, J. S., Lee, I. G., An, S. H., Shim, J. H. (2018). Synergistic effects of beta tri-calcium phosphate and porcine-derived decellularized bone extracellular matrix in 3D-printed polycaprolactone scaffold on bone regeneration. *Macromolecular Bioscience*, 18(6). Available from <https://doi.org/10.1002/mabi.201800025>.
- Kim, W., & Kim, G. (2020). Collagen/bioceramic-based composite bioink to fabricate a porous 3D hASCs-laden structure for bone tissue regeneration. *Biofabrication*, 12(1). Available from <https://doi.org/10.1088/1758-5090/ab436d>.
- Kim, W. J., Yun, H. S., & Kim, G. H. (2017). An innovative cell-laden α -TCP/collagen scaffold fabricated using a two-step printing process for potential application in regenerating hard tissues. *Scientific Reports*, 7 (1). Available from <https://doi.org/10.1038/s41598-017-03455-9>.
- Knoell, A. C. (1977). A mathematical model of an *in vitro* human mandible. *Journal of Biomechanics*. Available from [https://doi.org/10.1016/0021-9290\(77\)90054-9](https://doi.org/10.1016/0021-9290(77)90054-9).
- Koons, G. L., Diba, M., & Mikos, A. G. (2020). Materials design for bone-tissue engineering. *Nature Reviews Materials*, 5(8). Available from <https://doi.org/10.1038/s41578-020-0204-2>.
- Kopperdahl, D. L., & Keaveny, T. M. (1998). Yield strain behavior of trabecular bone. *Journal of Biomechanics*. Available from [https://doi.org/10.1016/S0021-9290\(98\)00057-8](https://doi.org/10.1016/S0021-9290(98)00057-8).
- Kumagai, H., Suzuki, T., Hamada, T., Sondang, P., Fujitani, M., & Nikawa, H. (1999). Occlusal force distribution on the dental arch during various levels of clenching. *Journal of Oral Rehabilitation*. Available from <https://doi.org/10.1046/j.1365-2842.1999.00473.x>.
- Lalloo, R., Lucchesi, L. R., Bisignano, C., Castle, C. D., Dingels, Z. V., Fox, J. T., James, S. L. (2019). Epidemiology of facial fractures: Incidence, prevalence and years lived with disability estimates from the Global Burden of Disease 2017 study. *Injury Prevention: Journal of the International Society for Child and Adolescent Injury Prevention*. Available from <https://doi.org/10.1136/injuryprev-2019-043297>.
- Laurencin, C. T., & El-Amin, S. F. (2008). Xenotransplantation in orthopaedic surgery. *The Journal of the American Academy of Orthopaedic Surgeons*, 16(1). Available from <https://doi.org/10.5435/00124635-200801000-00002>.

- Lee, D. J., Kwon, J., Kim, Y.-I., Wang, X., Wu, T.-J., Lee, Y.-T., Ko, C.-C. (2019). Effect of pore size in bone regeneration using polydopamine-laced hydroxyapatite collagen calcium silicate scaffolds fabricated by 3D mould printing technology. *Orthodontics & Craniofacial Research*, 22(S1). Available from <https://doi.org/10.1111/ocr.12261>.
- Lee, S., Choi, D., Shim, J. H., & Nam, W. (2020). Efficacy of three-dimensionally printed polycaprolactone/beta tricalcium phosphate scaffold on mandibular reconstruction. *Scientific Reports*, 10(1). Available from <https://doi.org/10.1038/s41598-020-61944-w>.
- Lee, S. J., Won, J.-E., Han, C., Yin, X. Y., Kim, H. K., Nah, H., Park, S. A. (2019). Development of a three-dimensionally printed scaffold grafted with bone forming peptide-1 for enhanced bone regeneration with *in vitro* and *in vivo* evaluations. *Journal of Colloid and Interface Science*, 539. Available from <https://doi.org/10.1016/j.jcis.2018.12.097>.
- Li, X., Wang, Y., Wang, Z., Qi, Y., Li, L., Zhang, P., Chen, X., & Huang, Y. (2018). Composite PLA/PEG/nHA/dexamethasone scaffold prepared by 3D printing for bone regeneration. *Macromolecular Bioscience*, 18(6). Available from <https://doi.org/10.1002/mabi.201800068>.
- Lopez, C. D., Diaz-Siso, J. R., Witek, L., Bekisz, J. M., Cronstein, B. N., Torroni, A., Coelho, P. G. (2018). Three dimensionally printed bioactive ceramic scaffold osseointegration across critical-sized mandibular defects. *The Journal of Surgical Research*, 223. Available from <https://doi.org/10.1016/j.jss.2017.100.027>.
- López-Jarana, P., Díaz-Castro, C. M., Falcão, A., Falcão, C., Ríos-Santos, J. V., & Herrero-Climent, M. (2018). Thickness of the buccal bone wall and root angulation in the maxilla and mandible: An approach to cone beam computed tomography. *BMC Oral Health*. Available from <https://doi.org/10.1186/s12903-018-0652-x>.
- Luo, Y., Chen, S., Shi, Y., & Ma, J. (2018). 3D printing of strontium-doped hydroxyapatite based composite scaffolds for repairing critical-sized rabbit calvarial defects. *Biomedical Materials (Bristol, England)*, 13(6). Available from <https://doi.org/10.1088/1748-605X/aad923>.
- Manson, P. N., Clark, N., Robertson, B., Slezak, S., Wheatly, M., Vander Kolk, C., & Iliff, N. (1999). Subunit principles in midface fractures: The importance of sagittal buttresses, soft-tissue reductions, and sequencing treatment of segmental fractures. *Plastic and Reconstructive Surgery*. Available from <https://doi.org/10.1097/00006534-199904040-00031>.
- Mantripragada, V. P., Lecka-Czernik, B., Ebraheim, N. A., & Jayasuriya, A. C. (2013). An overview of recent advances in designing orthopedic and craniofacial implants. *Journal of Biomedical Materials Research – Part A*, 101(11). Available from <https://doi.org/10.1002/jbm.a.34605>.
- Maroulakos, M., Kamperos, G., Tayebi, L., Halazonetis, D., & Ren, Y. (2019). Applications of 3D printing on craniofacial bone repair: A systematic review. *Journal of Dentistry*, 80. Available from <https://doi.org/10.1016/j.jdent.2018.110.004>.
- Miyowaki, S., Tanimoto, Y., Kawakami, T., Sugimura, M., & Takano-Yamamoto, T. (2001). Motion of the human mandibular condyle during mastication. *Journal of Dental Research*. Available from <https://doi.org/10.1177/00220345010800020701>.
- Moncal, K. K., et al. (2019). Collagen-infilled 3D printed scaffolds loaded with miR-148b-transfected bone marrow stem cells improve calvarial bone regeneration in rats. *Materials Science and Engineering: C*, 105. Available from <https://doi.org/10.1016/j.msec.2019.110128>.
- Moskalenko, Y., Weinstein, G., Kravchenko, T., Halvorson, P., Ryabchikova, N., & Andreev, J. (2012). The role of skull mechanics in mechanism of cerebral circulation. *Injury and Skeletal Biomechanics*. Available from <https://doi.org/10.5772/50887>.
- Motherway, J. A., Verschueren, P., Van der Perre, G., Vander Sloten, J., & Gilchrist, M. D. (2009). The mechanical properties of cranial bone: The effect of loading rate and cranial sampling position. *Journal of Biomechanics*. Available from <https://doi.org/10.1016/j.jbiomech.2009.050.030>.
- Muramatsu, K., Hashimoto, T., Tominaga, Y., & Taguchi, T. (2014). Vascularized bone graft for oncological reconstruction of the extremities: Review of the biological advantages. *Anticancer Research*, 34(6).

- Naeije, M., & Hofman, N. (2003). Biomechanics of the human temporomandibular joint during chewing. *Journal of Dental Research*. Available from <https://doi.org/10.1177/154405910308200708>.
- Nettleton, K., Luong, D., Kleinfehn, A. P., Savariau, L., Premanandan, C., & Becker, M. L. (2019). Molecular mass-dependent resorption and bone regeneration of 3D printed PPF scaffolds in a critical-sized rat cranial defect model. *Advanced Healthcare Materials*, 8(17). Available from <https://doi.org/10.1002/adhm.201900646>.
- Nyberg, E., Rindone, A., Dorafshar, A., & Grayson, W. L. (2017). Comparison of 3D-printed poly- ϵ -caprolactone scaffolds functionalized with tricalcium phosphate, hydroxyapatite, bio-oss, or decellularized bone matrix. *Tissue Engineering. Part A*, 23(11–12), 503–514. Available from <https://doi.org/10.1089/ten.tea.2016.0418>.
- Omar, O., et al. (2020). *In situ* bone regeneration of large cranial defects using synthetic ceramic implants with a tailored composition and design. *Proceedings of the National Academy of Sciences of the United States of America*, 117(43). Available from <https://doi.org/10.1073/pnas.2007635117>.
- Oppenheimer, A. J., Tong, L., & Buchman, S. R. (2008). Craniofacial bone grafting: Wolff's law revisited. *Cranio-maxillofacial Trauma & Reconstruction*, 1(1). Available from <https://doi.org/10.1055/s-0028-1098963>.
- Pae, H. C., et al. (2019). 3D-printed polycaprolactone scaffold mixed with β -tricalcium phosphate as a bone regenerative material in rabbit calvarial defects. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, 107(4). Available from <https://doi.org/10.1002/jbm.b.34218>.
- Pakdel, A. R., Whyne, C. M., & Fialkov, J. A. (2017). Structural biomechanics of the craniomaxillofacial skeleton under maximal masticatory loading: Inferences and critical analysis based on a validated computational model. *Journal of Plastic Reconstructive & Aesthetic Surgery*. Available from <https://doi.org/10.1016/j.bjps.2017.010.021>.
- Perez, J. R., Kouroupis, D., Li, D. J., Best, T. M., Kaplan, L., & Correa, D. (2018). Tissue engineering and cell-based therapies for fractures and bone defects. *Frontiers in Bioengineering and Biotechnology*, 6. Available from <https://doi.org/10.3389/fbioe.2018.00105>.
- Peterson, J., & Dechow, P. C. (2003). Material properties of the human cranial vault and zygoma. *The Anatomical Record. Part A, Discoveries in Molecular, Cellular, and Evolutionary Biology*. Available from <https://doi.org/10.1002/ar.a.10096>.
- Peterson, J., Wang, Q., & Dechow, P. C. (2006). Material properties of the dentate maxilla. *The Anatomical Record. Part A, Discoveries in Molecular, Cellular, and Evolutionary Biology*. Available from <https://doi.org/10.1002/ar.a.20358>.
- Pilipchuk, S. P., et al. (2016). Integration of 3D printed and micropatterned polycaprolactone scaffolds for guidance of oriented collagenous tissue formation *in vivo*. *Advanced Healthcare Materials*, 5(6). Available from <https://doi.org/10.1002/adhm.201500758>.
- Pogorelov, M., Husak, E., Solodivnik, A., & Zhdanov, S. (2017). Magnesium-based biodegradable alloys: Degradation, application, and alloying elements. *Interventional Medicine and Applied Science*, 9(1), 27–38.
- Pogrel, M. A., Podlesh, S., Anthony, J. P., & Alexander, J. (1997). A comparison of vascularized and nonvascularized bone grafts for reconstruction of mandibular continuity defects. *Journal of Oral and Maxillofacial Surgery*, 55(11), 1200–1206.
- Portigliatti Barbos, M., Bianco, P., Ascenzi, A., & Boyde, A. (1984). Collagen orientation in compact bone: II. Distribution of lamellae in the whole of the human femoral shaft with reference to its mechanical properties. *Metabolic Bone Disease & Related Research*, 5(6), 309–315. Available from [https://doi.org/10.1016/0021-8747\(84\)90018-3](https://doi.org/10.1016/0021-8747(84)90018-3).
- Pruim, G. J., de Jongh, H. J., & ten Bosch, J. J. (1980). Forces acting on the mandible during bilateral static bite at different bite force levels. *Journal of Biomechanics*. Available from [https://doi.org/10.1016/0021-9290\(80\)90237-7](https://doi.org/10.1016/0021-9290(80)90237-7).
- Qi, X., et al. (2017). Three dimensional printing of calcium sulfate and mesoporous bioactive glass scaffolds for improving bone regeneration *in vitro* and *in vivo*. *Scientific Reports*, 7. Available from <https://doi.org/10.1038/srep42556>.

- Rahaman, M. N., et al. (2011). Bioactive glass in tissue engineering. *Acta Biomaterialia*, 7(6). Available from <https://doi.org/10.1016/j.actbio.2011.030.016>.
- Reilly, D. T., & Burstein, A. H. (1975). The elastic and ultimate properties of compact bone tissue. *Journal of Biomechanics*, 8(6), 393–405. Available from [https://doi.org/10.1016/0021-9290\(76\)90178-0](https://doi.org/10.1016/0021-9290(76)90178-0).
- Rider, P., Kačarević, Ž. P., Alkildani, S., Retnasingh, S., Schnettler, R., & Barbeck, M. (2018). Additive manufacturing for guided bone regeneration: A perspective for alveolar ridge augmentation. *International Journal of Molecular Sciences*, 19(11). Available from <https://doi.org/10.3390/ijms19113308>.
- Rindone, A. N., Nyberg, E., & Grayson, W. L. (2018). 3D-printing composite polycaprolactone-decellularized bone matrix scaffolds for bone tissue engineering applications. *Methods in Molecular Biology*, 1577.
- Ross, C. F., et al. (2011). *In vivo* bone strain and finite-element modeling of the craniofacial haft in catarrhine primates. *Journal of Anatomy*. Available from <https://doi.org/10.1111/j.1469-7580.2010.01322.x>.
- Salzer, K. E., & Taylor, D. P. (1987). Bone grafts in craniofacial surgery. *Clinics in Plastic Surgery*, 14(1). Available from <https://doi.org/10.1055/s-0029-1215875>.
- Sarkar, N., & Bose, S. (2020). Controlled delivery of curcumin and vitamin K2 from hydroxyapatite-coated titanium implant for enhanced *in vitro* chemoprevention, osteogenesis, and *in vivo* osseointegration. *ACS Applied Materials & Interfaces*, 12(12), 13644–13656. Available from <https://doi.org/10.1021/acsami.9b22474>.
- Schwartz-Dabney, C. L., & Dechow, P. C. (2003). Variations in cortical material properties throughout the human dentate mandible. *American Journal of Physical Anthropology*. Available from <https://doi.org/10.1002/ajpa.10121>.
- Sheikh, Z., Hamdan, N., Ikeda, Y., Gryn timer, M., Ganss, B., & Glogauer, M. (2017). Natural graft tissues and synthetic biomaterials for periodontal and alveolar bone reconstructive applications: A review. *Biomaterials Research*, 21(1). Available from <https://doi.org/10.1186/s40824-017-0095-5>.
- Shim, J. H., et al. (2018). Porosity effect of 3D-printed polycaprolactone membranes on calvarial defect model for guided bone regeneration. *Biomedical Materials (Bristol, England)*, 13(1). Available from <https://doi.org/10.1088/1748-605X/aa9bbc>.
- Smolka, W., Eggensperger, N., Kollar, A., & Iizuka, T. (2005). Midfacial reconstruction using calvarial split bone grafts. *Archives of Otolaryngology – Head and Neck Surgery*, 131(2). Available from <https://doi.org/10.1001/archotol.131.20.131>.
- Song, Y., et al. (2020). Zinc silicate/nano-hydroxyapatite/collagen scaffolds promote angiogenesis and bone regeneration via the p38 MAPK pathway in activated monocytes. *ACS Applied Materials & Interfaces*, 12(14). Available from <https://doi.org/10.1021/acsami.0c00470>.
- Su, K., Zhou, Y., Hossaini-Zadeh, M., & Du, J. (2021). Effects of implant buccal distance on peri-implant strain: A Micro-CT based finite element analysis. *Journal of the Mechanical Behavior of Biomedical Materials*. Available from <https://doi.org/10.1016/j.jmbbm.2021.104325>.
- Sudhakar, K. N. V., Mohanty, R., & Singh, V. (2017). Evaluation of donor site morbidity associated with iliac crest bone harvest in oral and maxillofacial, reconstructive surgery. *Journal of Clinical and Diagnostic Research*, 11(6). Available from <https://doi.org/10.7860/JCDR/2017/28688.10053>.
- Sutradhar, A., Paulino, G. H., Miller, M. J., & Nguyen, T. H. (2010). Topological optimization for designing patient-specific large craniofacial segmental bone replacements. *Proceedings of the National Academy of Sciences of the United States of America*, 107(30), 13222–13227. Available from <https://doi.org/10.1073/pnas.1001208107>.
- Tcacencu, I., et al. (2018). Osseointegration of porous apatite-wollastonite and poly(lactic acid) composite structures created using 3D printing techniques. *Materials Science and Engineering C*, 90. Available from <https://doi.org/10.1016/j.msec.2018.040.022>.
- Tevlin, R., et al. (2014). Biomaterials for craniofacial bone engineering. *Journal of Dental Research*, 93(12). Available from <https://doi.org/10.1177/0022034514547271>.

- Thrivikraman, G., Athirasala, A., Twohig, C., Boda, S. K., & Bertassoni, L. E. (2017). Biomaterials for craniofacial bone regeneration. *Dental Clinics of North America*, 61(4). Available from <https://doi.org/10.1016/j.cden.2017.060.003>.
- Tollemar, V., Collier, Z. J., Mohammed, M. K., Lee, M. J., Ameer, G. A., & Reid, R. R. (2016). Stem cells, growth factors and scaffolds in craniofacial regenerative medicine. *Genes and Diseases*, 3(1). Available from <https://doi.org/10.1016/j.gendis.2015.090.004>.
- Trachtenberg, J. E., Placone, J. K., Smith, B. T., Fisher, J. P., & Mikos, A. G. (2017). Extrusion-based 3D printing of poly(propylene fumarate) scaffolds with hydroxyapatite gradients. *Journal of Biomaterials Science. Polymer Edition*, 28(6). Available from <https://doi.org/10.1080/09205063.2017.1286184>.
- Vaezi, M., et al. (2016). Characterization of new PEEK/HA composites with 3D HA network fabricated by extrusion freeforming. *Molecules (Basel, Switzerland)*, 21(6). Available from <https://doi.org/10.3390/molecules21060687>.
- Vallet-Regí, M. (2010). Evolution of bioceramics within the field of biomaterials. *Comptes Rendus Chimie*, 13(1–2). Available from <https://doi.org/10.1016/j.crci.2009.030.004>.
- Visscher, D. O., et al. (2016). Advances in bioprinting technologies for craniofacial reconstruction. *Trends in Biotechnology*, 34(9). Available from <https://doi.org/10.1016/j.tibtech.2016.040.001>.
- Wang, L., Xu, W., Chen, Y., & Wang, J. (2019). Alveolar bone repair of rhesus monkeys by using BMP-2 gene and mesenchymal stem cells loaded three-dimensional printed bioglass scaffold. *Scientific Reports*, 9(1). Available from <https://doi.org/10.1038/s41598-019-54551-x>.
- Wang, M. M., et al. (2019). Dipyridamole-loaded 3D-printed bioceramic scaffolds stimulate pediatric bone regeneration *in vivo* without disruption of craniofacial growth through facial maturity. *Scientific Reports*, 9(1). Available from <https://doi.org/10.1038/s41598-019-54726-6>.
- Winters, H. A. H., Kraak, J., Oosterhuis, J. W. A., & de Kleuver, M. D. (2013). Spinal reconstruction with free vascularised bone grafts; approaches and selection of acceptor vessels. *Scandinavian Journal of Surgery: SJS: Official Organ for the Finnish Surgical Society and the Scandinavian Surgical Society*, 102(1). Available from <https://doi.org/10.1177/145749691310200109>.
- Wolff, J. (1986). *The law of bone remodelling*. ...
- Xu, Z., et al. (2019). Poly(dopamine) coating on 3D-printed poly-lactic-co-glycolic acid/ β -tricalcium phosphate scaffolds for bone tissue engineering. *Molecules (Basel, Switzerland)*, 24(23). Available from <https://doi.org/10.3390/molecules24234397>.
- Yu, B. H., et al. (2020). Dynamic changes of facial skeletal fractures with time. *Scientific Reports*. Available from <https://doi.org/10.1038/s41598-020-60725-9>.
- Yu, J., Xu, Y., Li, S., Seifert, G. V., & Becker, M. L. (2017). Three-dimensional printing of nano hydroxyapatite/poly(ester urea) composite scaffolds with enhanced bioactivity. *Biomacromolecules*, 18(12). Available from <https://doi.org/10.1021/acs.biomac.7b01222>.
- Zeng, H., et al. (2020). Indirect selective laser sintering-printed microporous biphasic calcium phosphate scaffold promotes endogenous bone regeneration via activation of ERK1/2 signaling. *Biofabrication*, 12(2). Available from <https://doi.org/10.1088/1758-5090/ab78ed>.
- Zhang, Y., Wang, C., Fu, L., Ye, S., Wang, M., & Zhou, Y. (2019). Fabrication and application of novel porous scaffold *in situ*-loaded graphene oxide and osteogenic peptide by cryogenic 3D printing for repairing critical-sized bone defect. *Molecules (Basel, Switzerland)*, 24(9). Available from <https://doi.org/10.3390/molecules24091669>.
- Zhou, X., et al. (2016). Improved human bone marrow mesenchymal stem cell osteogenesis in 3D bioprinted tissue scaffolds with low intensity pulsed ultrasound stimulation. *Scientific Reports*, 6. Available from <https://doi.org/10.1038/srep32876>.
- Zhou, Y., Gong, C., Hossaini-Zadeh, M., & Du, J. (2019). *3D contact and strain in alveolar bone under tooth/implant loading*. Available from https://doi.org/10.1007/978-3-030-05861-6_77TMS 2019 148th annual meeting and exhibition supplemental proceedings. *Minerals, metals and materials series* (pp. 793–798). Springer International Publishing.

ADDITIVE MANUFACTURING FOR BONE LOAD BEARING APPLICATIONS

13

Mihaela Vlasea¹, Ahmad Basalah¹, Amir Azhari¹, Rita Kandel² and Ehsan Toyserkani¹

¹*Department of Mechanical and Mechatronics Engineering, University of Waterloo, Waterloo, ON, Canada*

²*Mount Sinai Hospital, University of Toronto, Toronto, ON, Canada*

13.1 NEED FOR BONE SUBSTITUTES

The incentive behind fabricating constructs with a direct application in bone and joint reconstruction surgeries lies in understanding the demand for such devices. Bone and cartilage conditions, such as arthritis, osteoporosis, traumatic musculoskeletal injuries, spinal injuries, and spinal deformities (Hutchinson, 2009), although mostly nonlife-threatening, can become very incapacitating, diminishing the quality of life of the affected individuals by causing ongoing pain, discomfort, inflammation, and restrictions in range of motion (Hutchinson, 2009). Furthermore, these conditions represent a major financial burden on the healthcare sector (Hutchinson, 2009; Cheng et al., 2013). The current conventional treatment for advanced joint and bone trauma is to fully or partially replace the affected area with tissue grafts (Gikas et al., 2009) or with artificial prosthetics (Bartel et al., 2006) to restore near-normal functions. Current state-of-the-art prosthetic implants fail to meet structural and functional requirements that would render them as permanent remediation solutions (Bartel et al., 2006). As a result, thousands of patients undergo painful and costly subsequent surgeries for implant replacements or readjustments. Cell-based or tissue graft solutions have been proven to ameliorate the quality of life of patients, but are limited in terms of size and anatomical shape of defect that can be addressed, as well as the availability of healthy donor tissue and morbidity of the donor site (Koh, 2004; Gikas et al., 2009; Vasiliadis et al., 2010). There is a pressing need for more successful bone and osteochondral reconstruction approaches that take into account biochemical, morphological, and anatomical factors. One such approach focuses on manufacturing biocompatible and/or bioresorbable bone substitutes with complex internal and external architecture and appropriate biochemical cues that can enhance or replace the defect area, gradually mature, and seamlessly integrate with the native tissue (Hutmacher, 2000). The bone substitute would serve as a biocompatible template that would encourage cell migration, proliferation, and differentiation, ideally acting as a temporary bioresorbable porous support until the bone matrix is regenerated (Bohner et al., 2011). A vast amount of work has been done in materials research and manufacturing methodologies in this field, specifically in constructing bone substitutes for load bearing applications; however, there is still a gap in understanding the ideal relationship between

the scaffold morphology (pore size, shape, and interconnectivity), transient biochemical interactions, and mechanical properties (Bohner et al., 2011; Butscher et al., 2011).

13.2 COMPOSITIONAL, STRUCTURAL AND MECHANICAL PROPERTIES OF BONE

13.2.1 COMPOSITIONAL PROPERTIES OF BONE AND REQUIREMENTS FOR BONE SUBSTITUTES

Bone is a dynamic and complex organ, encompassing a variety of tissues such as mineralized osseous tissue, cartilage, endosteum, periosteum, marrow, nerves, and blood vessels (Porter et al., 2009). The main role of the bone network is in providing the necessary mechanical support, movement, and protection, with other roles ranging from blood production, to storage of mineral materials, pH regulation, and housing multi progenitor cells (Porter et al., 2009; Szpalski et al., 2012). Due to the complex nature of the bone as biological system, in the context of fabricating bone substitute implants, the focus is generally on understanding the biochemical and structural makeup of the bone extracellular matrix (ECM), as well as the interaction of the ECM with cells and the environment in which they reside (Szpalski et al., 2012). The bone ECM is in essence a composite material comprised of carbonated apatite (~69% of the ECM), mainly hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) crystals, entrapped in an organic matrix (~22% of the ECM) of mostly type I collagen, and water (~9 % of the ECM) (Szpalski et al., 2012; Bose and Tarafder, 2012). Lipids, proteins, and osteogenic factors also reside in the ECM organic matrix (Bose and Tarafder, 2012). From a compositional point of view, the bone substitute matrix should be at least biocompatible with the ECM, cellular, and chemical environment, osteoconductive to encourage fast bone ingrowth from surrounding healthy tissue (Pilliar et al., 2001), as well as nontoxic, nonmutagenic, noncarcinogenic, and nonteratogenic (Leong et al., 2003). Ideally, the material should be osteoinductive to promote formation of new bone at the site (Porter et al., 2009; Yang et al., 2001).

13.2.2 STRUCTURAL PROPERTIES OF BONE AND REQUIREMENTS FOR BONE SUBSTITUTES

Structurally, the bone ECM is comprised of two main zones with very different morphological properties. Trabecular bone, also known as cancellous bone, is a highly porous bone matrix, with interconnected porosities between 50–90% and visible macropores in the range of 500–1000 μm (Karageorgiou and Kaplan, 2005). Trabecular bone has a complex and organized porous architecture, with trabeculae following the direction of mechanical stress (Porter et al., 2009) as a direct result of adaptations to mechanical loading, as postulated by Wolff's law (Bartel et al., 2006) and the mechanostat theory (Frost 2003). Trabecular bone encloses bone marrow and is enclosed by cortical bone. Cortical bone ECM has a compact solid-like structure, with enclosed vascular Haversian canals, having a low porosity between 3–12%, and pores <500 μm (Karageorgiou and Kaplan, 2005). Cortical bone has a solid structure with a series of voids, for example Haversian canals, with a 3–12% porosity (typical apparent density values for proximal tibial trabecular bone $0.30 \pm 0.10 \text{ g/cm}^3$). The bone ECM is constantly remodeled by the cells that reside in it, where osteoblasts are responsible for producing and mineralizing new bone matrix, osteocytes work on

Table 13.1 Range of mechanical properties for human cancellous and cortical bone (Porter et al. 2009; Yang et al. 2001)

Bone type	Tensile strength (MPa)	Compressive strength (MPa)	Young's modulus (GPa)
Cancellous bone	N/A	4–12	0.01–0.5
Cortical bone	60–160	130–225	3–30

maintaining the matrix, and osteoclasts are responsible for resorbing the matrix (Karageorgiou and Kaplan, 2005). From a structural standpoint, in designing a bone substitute, it is necessary to consider a gradient in porosity and mechanical properties, from a dense external configuration matching the characteristics of cortical bone to the highly porous region with interconnected porosity matching the characteristics of cancellous bone (Mehrali et al., 2013). This means that an ideal bone substitute must have a heterogeneous porous structure, with varying physical and mechanical characteristics. In addition, the implant must also be designed to have an anatomically accurate three-dimensional shape in order to maintain a natural contact load distribution post implantation (Koh, 2004).

13.2.3 MECHANICAL PROPERTIES OF BONE AND REQUIREMENTS FOR BONE SUBSTITUTES

The high level of porosity and pore interconnectivity that is ideal for a bone substitute may be limited by the mechanical strength requirements for that specific implant, especially in the case of load bearing applications. The bone substitute should provide physical support, starting from the seeding process *in vitro* until the tissue is remodeled *in vivo*. Furthermore, the implant must provide sufficient mechanical support to endure *in vivo* stresses and load bearing cycles (Hutmacher, 2000). Table 13.1 summarizes the range in mechanical properties of human cancellous and cortical bone.

13.3 DIFFICULTIES IN ACHIEVING AN IDEAL BONE SUBSTITUTE

Manufacturing of optimal porous bone substitutes from a biochemical, structural, and mechanical properties point of view is highly complex due to a collection of factors. From an architectural standpoint, the bone substitute supports biological and mechanical functions (Bohner et al., 2011), which may be in conflict. For example, for increasing the load-bearing property of the material, a denser material is needed, which conflicts with the requirement of having a highly porous matrix to encourage bone ingrowth and fluid permeability (Karageorgiou and Kaplan, 2005). What is generally defined as an optimization of scaffold properties is likely a tuning of a single parameter with little regard to how other scaffold properties are modified. Furthermore, characterizing, digitizing, and manufacturing the scaffold architecture are difficult tasks. Using characterization methods to reveal pore surface, pore volume, pore shape, interconnectivity, and volume porosity in bone tissues (Bohner et al., 2011), and furthermore translating such data into a digital format that can be interpreted into fabrication methodologies in a continuous or discrete fashion can be a challenge. Typically, the

interconnected macroporosity should be $>50\text{ }\mu\text{m}$ (Bohner et al., 2011; Yang et al., 2001; Chang et al., 2000; Kujala et al., 2003; Karageorgiou and Kaplan, 2005; Bose and Tarafder, 2012), with a specific orientation to match the stress loading conditions and fluid and nutrient transport mechanics (Bohner et al., 2011). Also, what is defined as microporosity, with a diameter ranging between $0.1\text{--}10\text{ }\mu\text{m}$ (Bohner et al., 2011; Karageorgiou and Kaplan, 2005; Bose and Tarafder, 2012) has shown an effect on the biological response of scaffolds, thus the pores at this scale should be characterized and integrated in the final design. From a structural standpoint, it is also necessary to implement a gradient in porosity and mechanical properties, from a dense external configuration matching the characteristics of cortical bone to the highly porous region with interconnected porosity matching the characteristics of cancellous bone (Mehrali et al., 2013; Butscher et al., 2011; Porter et al., 2009). Manufacturing methodologies that can incorporate the interpretation and implementation of digital data at a macro- and micropore scale are of concern.

Another issue related to manufacturing the appropriate bone substitute architecture lies in the development of appropriate software interpreter design strategies (Lin et al., 2004; Cai and Xi, 2008; Sanz-Herrera et al., 2009) that can convert the desired structural porous morphology and mechanical properties of the bone to be replaced, into appropriate voxel units that can be fabricated using various manufacturing platforms. Such voxels may be computed mathematically using topology optimization algorithms (Lin et al., 2004; Hollister, 2005) or numerical simulation (Sanz-Herrera et al., 2009).

From a material standpoint, difficulties arise in designing structures that can bioresorb *in vivo* at an appropriate rate matching bone remodeling. In the context of regenerative medicine, the terminology of materials with biodegradable, bioresorbable, bioerodible, and bioabsorbable (Hutmacher, 2000) properties are often used. The biodegradation pathway will have an effect on the mechanical, structural, and biochemical properties of the scaffold, and needs to be fully understood (Bohner et al., 2011). Some of the parameters that affect the degradation rate are pore size, pore interconnectivity, permeability, scaffold shape, and volume, as well as implantation location within the musculoskeletal system. Furthermore, the long-term native tissue response to the degradation products should also be considered (Bohner et al., 2011). To add to the difficulty of producing an ideal implant, the overall biochemical, structural, and mechanical properties of the bone substitute should match patient-specific needs such as age, gender, health, metabolism, implant location, and loading conditions (Bohner et al., 2011).

13.4 METALLIC BONE SUBSTITUTES

13.4.1 METALLIC MATERIALS, LIMITATIONS AND OPPORTUNITIES

For a long time, metals were the main material utilized for orthopedic implants. This interest in metals resulted from the excellent physical and mechanical properties that are intrinsic to metals. At present, the interest in nonmetallic materials has prompted the fabrication of tissue scaffolds. These materials are mostly polymer or ceramic, and are used to produce biodegradable scaffolds. Biodegradable scaffolds can be useful for young patients, because they have high growth rates of tissue to restore the functionality of the damaged area. However, the case is completely different for senior citizens, who have very low tissue growth rates. When faced with a certain degradation rate of the materials, this may cause a mismatch in terms of the mechanical properties (Yarlagadda et al., 2005). Therefore, permanent metallic bone substitutes are more appropriate in the case of older patients. Several metals have been

Table 13.2 Comparison of the mechanical properties of different metals (Krishna et al. 2007)

Material	Bone	Magnesium	Co–Cr–Mo and alloys	Ti and alloys	Stainless steels
Density (g cc ⁻¹)	1.8–2.1	3.1	8.3–9.2	4.4–4.5	7.9–81
Compressive strength (MPa)	130–180	65–100	450–1896	590–1117	170–310
Elastic modulus (GPa)	3–20	41–45	200–253	55–117	189–205
Toughness (MPam ^{1/2})	3–6	15–40	100	55–115	50–200

used for implants, such as stainless steels (316L), Co–Cr–Mo, pure titanium, titanium alloys, and tantalum. Each metal has advantages and disadvantages that can either expand or limit its usage.

Stainless steel is considered one of the first metals used in the orthopedic field as plates and screws for bone fixation in the early twentieth century (Stevens et al., 2008). The most popular stainless steel alloy used in prosthesis fixation is (316L), with moderate strength and toughness in comparison to other metals as shown in Table 13.2. This alloy is distinguished by good corrosion resistance in comparison to other steel alloys, since the 12% Cr in its content forms a corrosion protective layer Cr₂O₃ on the surface (Navarro et al., 2008). This metal is both widely available and economically effective in terms of processing and manufacturing (Long and Rack, 1998; Dabrowski et al., 2010). However, as the wear resistance of stainless steel is very low, its usage in hip replacement was stopped (Navarro et al., 2008). Nowadays, stainless steel is rarely used for orthopedic implants. Instead, stainless steel is used for temporary fixation devices such as nails, screws, and plates due to the superiority of other metals such as Ti, Ti alloys, and Co–Cr alloy in terms of mechanical properties and corrosion resistance (Navarro et al., 2008).

Another widely used metal in the orthopedic industry is Co–Cr–Mo alloy that is characterized by a high level of mechanical strength, fatigue strength, wear resistance, and low cost of production (Reclaru et al., 2005; Navarro et al., 2008; Dabrowski et al., 2010). While these advantages are considered beneficial for some applications, this is not generally true for orthopedic implants, since this metal's high strength and elastic modulus has caused a stress shielding between the implant and the bone due to the mismatch of mechanical properties. Moreover, the high level of metal ions that are released and the nanoparticle debris which is caused by the wear, negatively affect the biocompatibility of this metal (Billi and Campbell, 2010; Dabrowski et al., 2010). Despite these drawbacks, this metal is the most preferable in hip replacement due to its high level of wear resistance compared to the other metals. In particular, this material is used to fabricate the femoral head and acetabular cup which is an area of high friction (Navarro et al., 2008). In addition, dentists have some interest in this metal as a coating for some dental devices (Long and Rack, 1998).

NiTi (nitinol) is a shape memory alloy, which means it can restore its original shape after a plastic deformation by using a heat treatment. This alloy was introduced in 1960 and is characterized by high strength, superelasticity, and good corrosion resistance (Shishkovsky et al., 2008; Michiardi et al., 2006; Navarro et al., 2008). However, the release of the Ni ions limits its usage as an orthopedic prosthesis due to the possibility of toxicity and the inflammation effect of the surrounding tissue. This has led researchers to treat the surface through exposure to the oxidation

process in an attempt to create a protective layer free of Ni (Wataha et al., 2001; Navarro et al., 2008; Michiardi et al., 2006).

Alternatively, degradable metals have captured the interest of some researchers. One of these metals is magnesium (Mg), which is characterized by having low elastic modulus. In addition, Mg is an osteoconductive material and it does not show any inflammatory effect following fixation (Staiger et al., 2006). However, as Mg is a degradable material, the dissolved particles may cause toxicity, which could limit its usage in orthopedic applications (Alvarez and Nakajima, 2009).

Titanium (Ti) was introduced in the orthopedic field in 1965 in the form of screws and plates. This long period of usage illustrates the preference of using Ti, as supported by long-term clinical data, which indicates this material's good biocompatibility. Moreover, Ti is characterized by a nano-oxide layer covering its surface that increases *in vivo* osteointegration (Frosch and Stürmer, 2006). Indeed, from a biological aspect, Ti has proved its compatibility more so than stainless steel or CoCr, as proven by the cell culturing of these metals (Frosch & Stürmer, 2006). Overall, Ti is considered one of the strongest and highest corrosion-resistant metals. Moreover, Ti is characterized by an excellent strength-to-weight ratio and good toughness (Ryan et al., 2006; Navarro et al., 2008).

Recently, it has been noted that the science community is concentrating on Ti alloys, in particular, Ti-6Al-4V, to fabricate bone substitute scaffolds. This interest originates from the good mechanical properties and biocompatibility of this alloy. The presence of aluminum (Al) and vanadium (V) in this alloy improves the mechanical properties compared to commercially pure Ti (CP Ti) (Navarro et al., 2008). One of the most important benefits of Ti and its alloy (Ti-6Al-4V) is its relatively low elastic modulus which is the half of the Co—Cr modulus, which could assist in reducing the effect of the stress shielding (Ryan et al., 2006). Despite the fact that Ti has a low elastic modulus in comparison to other metals, the mismatch between bone and implant still exists (Ryan et al., 2006). Arguably, the presence of V in this alloys is a source of concern, since V is toxic (Okazaki, 2001; Alvarez and Nakajima, 2009). Further drawbacks of Ti and its alloys include the expensive machining cost and the sophisticated heat treatment process (Navarro et al., 2008). Nowadays, most dental implants are fabricated from CP Ti, and Ti-6Al-4V are used in orthopedic applications (Navarro et al., 2008).

In order to eliminate the effect of V in Ti6Al4V alloy, new Ti alloys have been developed to overcome the toxicity issue, such as Ti-6Al-7Nb, Ti-5Al-2.5Fe, Ti-35Nb-5Ta-7Zr, Ti-35Nb-5Ta-7Zr-0.4O, and Ti-15Zr-4Nb-4Ta. For instance, Ti-15Zr-4Nb-4Ta has shown excellent mechanical properties, biocompatibility, and good corrosion resistance. In addition, it is likely that more bone was formed than that which formed around the Ti 6Al 4V when implanted on the bone marrow of the rat tibia (Okazaki, 2001). Most of these alloys are still in the development stage in order to characterize the mechanical properties and biocompatibility for application in bone regenerative medicine (Navarro et al., 2008).

Another material, tantalum (Ta) is considered one of the best metals in the orthopedic field due to its fairly low Young's modulus, high corrosion resistance, and excellent biocompatibility (Matassi et al., 2013). A recent study comparing Ta and Ti showed that Ta is superior compared to Ti in regards to cell integration with a controllable Young's modulus in the range of 1.5–20 GPa by changing the porosity of the structure (Balla et al., 2010). However, the high cost of production and fabrication of this metal is the main obstacle limiting its usage in the orthopedic industry along with short-term clinical data (Dabrowski et al., 2010; Matassi et al., 2013).

Generally, the major constraints which can govern the success of a load bearing implant are biocompatibility, porosity, and proper mechanical properties. The highly important mechanical properties in the designing of a load bearing implant are Young's modulus and compressive

strength. Other mechanical properties such as bending strength are less relevant in load bearing implant. However, certain properties such as fatigue strength are necessary for a long lasting implant, but are not as crucial as Young's modulus and compressive strength, since these properties may cause short-term failure of implant (Oh et al., 2003).

13.4.2 AM OF METALS FOR BONE SUBSTITUTES

Historically, conventional techniques were used to fabricate porous metals for several industrial applications. Once porous metal was introduced in early 1972 in the orthopedic field (Weber and White, 1972), scientists looked for ways to control and improve the properties of this metal foam. Consequently, several conventional techniques emerged. However, these techniques are not capable of precisely controlling the porosity and producing a scaffold with a predefined microstructure. Also, these techniques cannot produce a scaffold with a contoured external shape. In order to overcome these obstacles, additive manufacturing (AM) techniques are considered the most promising alternative for precise fabrication of internal and external features of scaffold architecture.

There are many AM techniques that have been used in the fabrication of metal scaffolds, such as three-dimensional printing (3DP), electron beam melting (EBM), selective laser melting (SLM), direct metal deposition (DMD), and selective laser sintering (SLS) (Ryan et al., 2008; Murr et al., 2009; Mullen et al., 2009; Dinda et al., 2008; Shishkovsky et al., 2008). Each technique has different opportunities and limitations. Some of these techniques rely on laser technology, and others rely on the injection of the slurry. The costs of production through these techniques are varied according to complexity. For example, the laser sintering methods are highly expensive due to their use of laser technology and the capability of producing a precise microstructure. Other techniques appropriate for fabricating a scaffold have acceptable microstructure details, such as fiber deposition and 3DP.

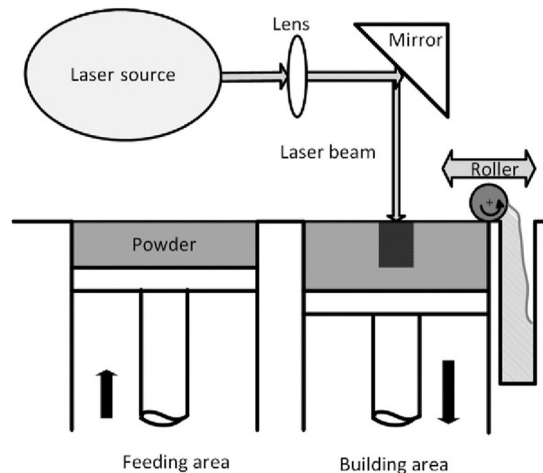


FIGURE 13.1

Schematic presentation of working principle of SLS technique.

SLS is an AM technique (Figure 13.1) that works by sintering very fine layers of metal powders layer over layer using a CO₂ laser beam, either directly or indirectly. In direct SLS, the powder mixture is a compound of two metals: a low sintering temperature metal and the main metal (Campanelli et al., 1994). In this technique, the laser beam melts the low sintering temperature metal and uses it to bind the main metal particles to each other. For instance, in a study where NiTi dental implant was fabricated using DMLS, the base metal was Ti and the binding agent was Ni (Shishkovsky et al., 2008). The second technique is called indirect SLS, where a preprocessing of the powder is required to coat the metal powder particles with some polymer to work as a binding agent of the green sample. This technique requires a postprocessing of the green sample by debinding the polymer from the green sample followed by the sintering of the metal particles in a shielded environment at a very high temperature. The diversity of materials produced by this manufacturing method is one of the most important advantages of this technique (Wong and Hernandez, 2012a). The final product resolution is moderate since heat transfers to the adjacent area and fuses extra particles to the targeted area, which might lead to a limitation in the accuracy according to the size of particles (Woesz, 2008a; Wong and Hernandez, 2012b). Economically speaking, fabrication using SLS is costly (Dabrowski et al., 2010). Technically, SLS is considered a lengthy manufacturing method, since it requires preprocessing of the powder and the laser sintering process is time-consuming. In addition, the resulting product may require a postprocessing heat treatment that usually takes hours to complete (Campanelli et al., 1994; Woesz, 2008b).

Another complex technique, EBM, works based on the layering process similar to SLS. EBM differs from SLS in its use of an electron beam rather than a laser to sinter the powders, which is generated in a tungsten filament and is accelerated and controlled with a magnetic field (van Noort 2012). This manufacturing technique is relatively fast and the building of samples takes place under vacuum (Alvarez and Nakajima, 2009). It offers an attractive opportunity in the fabrication of fully dense or porous parts due to several controllable parameters such as beam current, scan rate, and sequence variations (Murr et al., 2009). In this technique, an additive or fluxing agent is not required to fulfill the melting process because the electron beam is powerful enough to raise the temperature of the particles to the melting point (van Noort 2012). However, there are still some limitations in this method such as the low dimensional accuracy and surface quality due to shrinkage. Additionally, this technique is costly, and removing the excess material inside the structure can prove difficult (Wen et al., 2002). Different metals have been fabricated using EBM, such as Cp-Ti, Ti-6Al-4V, and Co/Cr for orthopedic implant and maxillofacial surgery (Alvarez & Nakajima 2009; van Noort, 2012).

Similarly, SLM utilizes the same mechanism of the EBM, and a wide range of materials in powder form can be used (Alvarez and Nakajima, 2009). Moreover, SLM is able to produce solid and porous parts based on the laser energy density (Mullen et al., 2009). Also, this technique is free of binders and fluxing agents, so there is no need for a postprocessing step (Pupo et al., 2013; Alvarez and Nakajima, 2009; Campanelli et al., 1994). Unlike EBM, SLM uses an ytterbium fiber laser 200 W power and uses Ar or N in the building chamber, which may increase the thermal conductivity and maintain a consistent rapid cooling of the printed zone more than EBM (Murr et al., 2012; Mullen et al., 2009). However, high production cost, lengthy fabrication time, and difficulty in removing the trapped powder are the major limitations of this technique (Dabrowski et al., 2010; Alvarez and Nakajima, 2009; Campanelli et al., 1994). Furthermore, the vaporization phenomenon is one of the drawbacks of this technique. This phenomenon is generated due to the high temperature of the molten pool caused by the laser beam evaporating the particles. This leads to an overpressure in the molten pool, which results in spewing of some

molten metals out of the pool (Campanelli et al., 1994). Today, this technique is utilized in the aerospace industry, orthopedics prostheses, and dental implants (Pupo et al. 2013).

In the same context, laser engineering net shaping (LENS) and direct metal deposition (DMD) are both AM techniques used in manufacturing bone substitutes. These techniques mainly depend on laser technology to fuse the metal powder. LENS systems use a neodymium–yttrium–aluminum–garnet (Nd:YAG) laser, while DMD uses the CO₂ laser beam. The powder feeding systems are completely different, since the powder feeding in the DMD comes through a concentric ring at the tip of the laser nozzle, while in the LENS the powder comes through different powder feeders to the melting zone. Both techniques use an inert gas during the manufacturing process to avoid oxidation (Dinda et al., 2008). With these techniques, it is possible to use a wide range of metal powders such as stainless steel, nickel-based alloys, Ti and its alloys, tooling steel, copper alloys, alumina, or a combination of these (Lin et al., 2009; Wong and Hernandez, 2012b; Woesz, 2008b). Thus, LENS can be useful for the repairing or re-manufacturing process that cannot be implemented by other AM techniques (Wong & Hernandez, 2012b). LENS and DMD work through the process of depositing the metal layer-over-layer. Both are similar in terms of processing; however, the high cost of DMD has boosted LENS's popularity. The need for support structures for overhanging features is one of the limitations of the use of these techniques in the fabrication of orthopedic prostheses (Woesz, 2008b). Another drawback is the residual stress caused by the rapid heating and solidifying process (Wong and Hernandez, 2012b).

Many studies have been conducted using two techniques: fiber deposition (FD) and 3DP. The two have been chosen mainly due to their manufacturing simplicity compared with SLS or EBM and cost efficiency. The base material of FD is metal powder. In the FD method, the powders are mixed with a solution to form the slurry which can be deposited from the machine onto a substrate. The scaffold is built by the process of layering from bottom to top. The FD technique requires postprocessing of the product by the sintering of the produced scaffold in a very high temperature furnace. When using FD in the fabrication of a scaffold, several parameters can influence the strength and the porosity of the structure, such as the gap between fibers, the fiber's lay down angle, and the nozzle's diameter (P. J. Li et al., 2007; Li et al., 2005). The dimensional accuracy of the final product is poor compared to other techniques such as

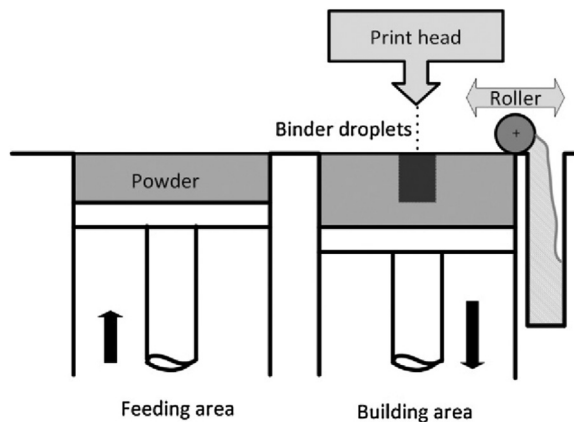


FIGURE 13.2

Simple sketch showing the working principle of the 3DP machine.

3DP due to the wet nature of the fiber slurry, which leaves a high level of shrinkage after drying and sintering (Basalah et al., 2012). In fact, this technique is still in the experimental stage.

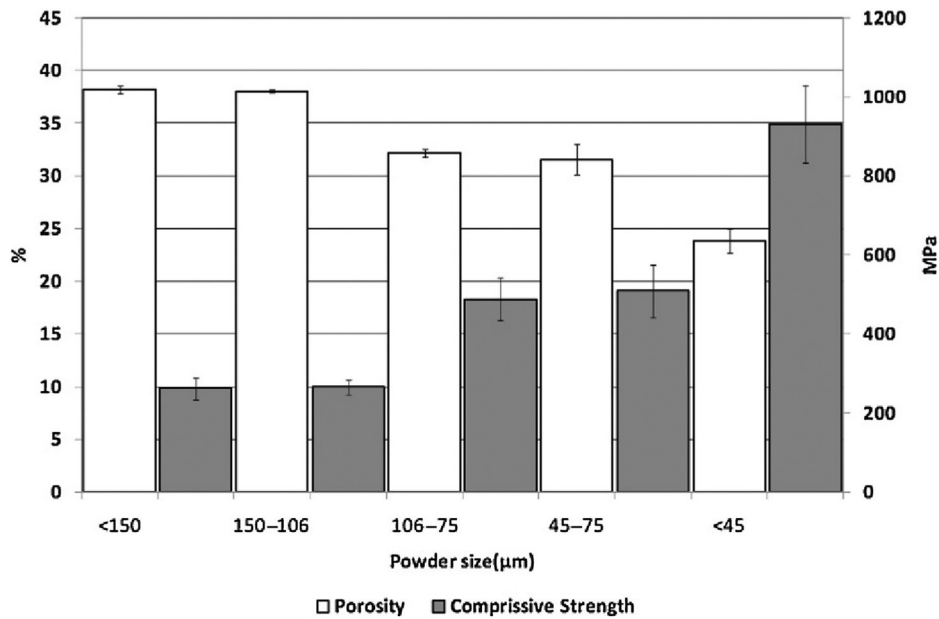
One of the simplest rapid prototyping techniques is the ink-jet-based 3DP, as shown in Figure 13.2. In this technique, the printer functionality is similar to an ink-jet printer, where instead of ink, a binder is dispersed through a print head on each layer of powder. Similar to previous methods, this printer works on a layer-by-layer basis, starting from the bottom and going up. The printer has two different powder zones: one for building the structure and the other for feeding the powder, so that when the feeding area goes up, the building area lowers down, moving a distance equivalent to the required layer thickness. A counter-rotating roller spreads the powder from the feeding bed over to the building bed, followed by the injection of the binder based on an image corresponding with the slice layer data of the 3D structure. This technique is very fast in the building process and is significantly cheaper than other techniques (Woesz, 2008b). This machine was used indirectly to produce a porous metal scaffold by printing a sacrificial mold from alumina powder to cast the Co-Cr alloy which is then removed through several thermal and chemical steps (Curodeau et al., 2000). Similarly, the same principle has been used to create a Ti scaffold and wax template as an alternative sacrificial mold (Ryan et al., 2008). This method of manufacturing provides a precise surface texture. However, the time-consuming process through the multiple stages of manufacturing is considered to be the main disadvantage of the indirect 3DP technique. This attempt has fascinated and motivated many researchers to replicate this success through direct printing of the desired metal scaffold, followed by sintering of the green part under a high temperature vacuum furnace (Hutmacher et al., 2004; Basalah et al., 2012). This technique has fewer stages than indirect printing. With 3DP, several parameters are able to form the microstructure of the desired part such as powder size, sintering temperature, and duration (Basalah et al., 2012). The layer thickness is primarily chosen based on the particle size and cannot be thinner than the largest particle in the powder (Hutmacher et al., 2004). However, the resolution is lower than the laser-based techniques because the binder penetrates the powders adjacent to the targeted area (Woesz, 2008b). Difficulties in removing the trapped powder from the green part are the main disadvantage of this technique (Hutmacher et al., 2004; Woesz, 2008b; Basalah et al., 2012).

Using the 3DP method and CP Ti as a building material, the authors' group has been able to develop a set of optimized processing conditions to assure control over a microstructure and the associated mechanical and physical properties of the structure (Basalah et al., 2012). Material powder size is one of the most influential parameters on changing the strength and density of the structure, as shown in Figure 13.3. Also, the physical appearance of the final product is crucial in the fabrication of the orthopedic implant, since a high level of shrinkage in the implant is undesirable, and the particle size influences the shrinkage, as shown in Figure 13.4.

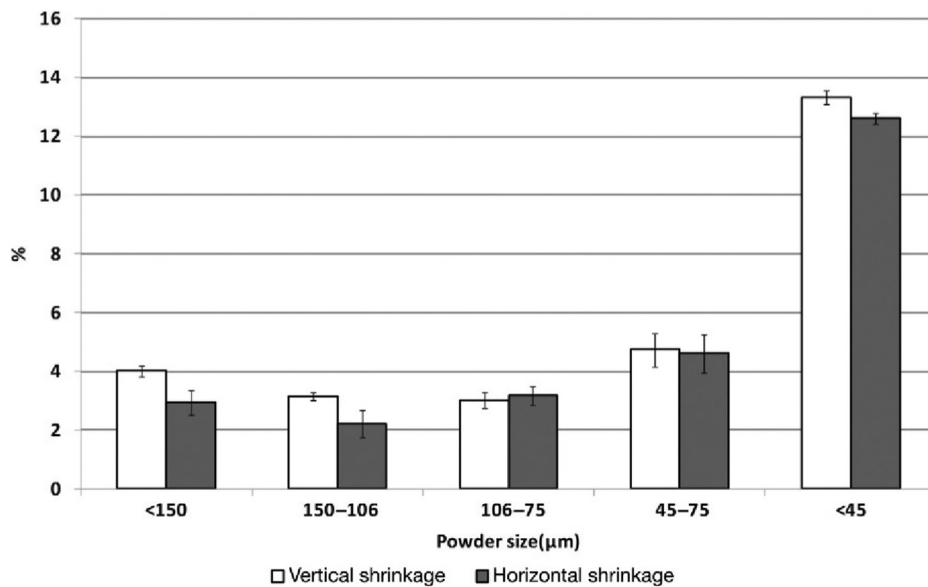
As a whole, AM techniques offer a good control over the manufacturing of the external and internal details of metallic bone substitute scaffolds, with limitations in the creation of complex internal interconnected macro pores (Yarlagadda et al., 2005).

13.5 BIO-CERAMIC BONE SUBSTITUTES

The appropriate materials for constructing bone substitutes for tissue regeneration or augmentation should adhere to a basic set of criteria stating that: (i) the material must be biocompatible to avoid the expression of unwanted immune responses, and must be osteoconductive to encourage rapid integration with

**FIGURE 13.3**

Compressive strength and porosity of the porous structures fabricated by varied sizes of Ti powder.

**FIGURE 13.4**

Vertical and horizontal shrinkage of Ti samples fabricated by varied sizes of Ti powder.

surrounding bone, and ideally osteoinductive to promote formation of new bone at the site (Porter et al., 2009; Yang et al., 2001); (ii) the material should be bioresorbable such that it is capable of bulk degradation and resorption through natural pathways (Hutmacher, 2000; Porter et al., 2009; Bohnert et al., 2012); and (iii) the material should provide the physical support, starting from the seeding process *in vitro* until the tissue is remodeled *in vivo*, to support stresses and load bearing cycles (Hutmacher, 2000; Bohnert et al., 2012; Bose and Tarafder, 2012). Bioceramics, such as crystalline ceramics, amorphous glasses, and ceramic composites, have been studied in the context of regenerative medicine as bone substitute materials as they show promise in meeting the outlined basic material criteria.

13.5.1 BIOCERAMIC, BIOACTIVE GLASSES AND COMPOSITE MATERIALS

Depending on the chemical composition and porous architecture of the bioceramic material, the main resorption mechanisms reported in literature can be classified as chemical dissolution, cell-mediated dissolution, hydrolysis, or enzymatic decomposition (Bohner et al., 2012), where resorption is understood here as the gradual removal of material from the bone substitute construct over time. To select an appropriate bioceramic for a bone substitute, it is important to understand the resorption mechanism, as well as the link between the rate of resorption and the rate of bone formation to ensure mechanical stability of the implant during bone remodeling. One of the most important resorption pathways is cell-mediated dissolution, since it is a biologically controlled mechanism, enabling the rate of degradation of the underlying bone substitute to be controlled by the host cells (Bohner et al., 2012; Detsch et al., 2010). Cell-mediated dissolution of materials is ideally validated *in vivo*, however *in vitro* tests can be done to observe so-called “dissolution” pits produced by osteoclast or osteoclast-like cells on the material (Bohner et al., 2012).

Calcium phosphates bioceramics are very promising materials in producing bone substitute components, as it was found that the degradation of these ceramics produces calcium and phosphate ions, which regulate bone metabolism, as they promote new bone formation through osteoinduction (Bohner et al., 2012; Bohner, 2010). For calcium phosphates, the main *in vivo* resorption is done through cell-mediated dissolution, also called osteoclastic resorption, where the osteoclast cells in contact with the material release hydrochloric acid in small amounts, enough to lower the local pH at the site, triggering calcium phosphate dissolution (Detsch et al., 2010; Bohner, 2010). Generally, calcium ions influence osteoblast proliferation and osteoclast regulation, while phosphate ions regulate osteoblast apoptosis and mineralization rate (Shanjani, 2011; Bohner et al., 2012). *In vitro*, the degradation of calcium phosphates in aqueous solutions generally occurs through chemical dissolution (Pilliar et al., 2001) and the rate of degradation is influenced mainly by the crystallinity of the material, solubility, porosity, surface area, and pH of the solution (Shanjani, 2011). There is a range of calcium phosphates ceramics, depending on the molar ratio of Ca:P, with different resorption properties, as summarized in Table 13.3. Calcium phosphates are popular materials in bone regenerative medicine as they have great versatility in terms of biodegradability and bioactivity, and they have chemical similarity to bone minerals (Bose and Tarafder, 2012).

Hydroxyapatite (HA) is a calcium phosphate compound with a strong compositional similarity to bone (Bohner et al., 2012; Bose and Tarafder, 2012), and with a proven osteoinductive and osteoconductive properties (Bose and Tarafder, 2012), forming strong bonds with the surrounding bone tissue. As a biomaterial in regenerative medicine, HA has been found to have the lowest resorption rate amongst calcium phosphates (Gbureck, et al., 2007), in the order of years, causing

Table 13.3 Bioceramics and bioactive glasses for bone substitutes

Class	Material name	Chemical formula	Main resorption	References
Calcium Phosphates	Calcium polyphosphate (CPP)	$[\text{Ca}(\text{PO}_3)_2]_n$	Cell mediated dissolution (moderate)	(a)
	Dicalcium phosphate (DCP)	CaHPO_4	Chemical dissolution (fast)	(b)
	Dicalcium phosphate dihydrate (DCPD)	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	Chemical dissolution (fast)	(c)
	Octacalcium phosphate (OCP)	$\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$	Cell mediated dissolution (slow)	(d)
	Tricalcium phosphate (TCP)	$\text{Ca}_3(\text{PO}_4)_2$	Cell mediated dissolution (moderate)	(e)
	Hydroxyapatite (HA)	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	Cell mediated dissolution (slow)	(f)
	Tetracalcium phosphate (TTCP)	$\text{Ca}_4\text{P}_2\text{O}_9$	Cell mediated dissolution (moderate)	(g)
Calcium Carbonate	Calcium carbonate	CaCO_3	Chemical dissolution or cell mediated	(h)
Calcium Sulfates	Hemihydrate (Plaster of Paris)	$\text{CaSO}_4 \cdot 0.5\text{H}_2\text{O}$	Chemical dissolution	(i)
	Dihydrate (Gypsum)	$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$	Chemical dissolution	(i)
Bioactive Glasses	Bioglass 45S5	Na_2O (24.5%) CaO (24.5%) SiO_2 (45%) P_2O_5 (6%)	Partial dissolution (very low)	(j)
	Bioglass 58S	CaO (36%) SiO_2 (60%) P_2O_5 (4%)	Partial dissolution (low)	(k)
	Borate glass 13-93B3	Na_2O (5.8%) K_2O (11.7%) MgO (4.9%) CaO (19.5%) SiO_2 (34.4%) B_2O_3 (19.9%)	Partial dissolution (low)	(l)
	Phosphate glass $\text{P}_{50}\text{C}_{35}\text{N}_{15}$	Na_2O (9.3%) CaO (19.7%) P_2O_5 (71.0%)	Partial dissolution (moderate)	(m)

(a) (Pilliar et al., 2013; Kandel et al., 2006; Grynpas et al., 2002; Pilliar et al., 2007; Shanjani et al., 2013) (b) (Gbureck, Hölzel, Klammert, et al., 2007); (c) (Gbureck, Hölzel, Klammert, et al. 2007; Alge et al., 2012); (d) (Anada et al., 2008); (e) (Kim et al., 2014; Friesenbichler et al., 2014); (f) (Bohner et al., 2012; Bose and Tarafder, 2012); (g) (Moseke and Gbureck, 2010); (h) (Bohner, 2010; Bohner et al., 2012); (i) (Bohner, 2010); (j) (Rahaman et al., 2011; Gerhardt and Boccaccini, 2010); (k) (Pereira et al., 2014); (l) (Rahaman et al., 2011; Gerhardt and Boccaccini, 2010); (m) (Rahaman et al., 2011; Gerhardt and Boccaccini, 2010)

mechanical incompatibility at the implantation site (Bohner et al., 2012). To mitigate the slow degradation rate, HA is typically used in conjunction with other faster-resorbing calcium phosphates to produce so-called biphasic materials, or with polymers to produce composites. For example, mixtures of HA with tricalcium phosphate (β -TCP) are commonly used commercially as bone fillers (Bone Save, Stryker, 80%TCP, 20% HA) and experimentally in literature, where ceramic mixtures of HA and β -TCP (Bose and Tarafder, 2012; Sánchez-Salcedo et al., 2008; Maté-Sánchez de Val

et al., 2014; Kobayashi and Murakoshi, 2014) or β -TCP and nanoscale HA (Shuai et al., 2013) were used to take advantage of the intrinsic strength provided by HA, and the resorbable properties of β -TCP. Octacalcium phosphate (OCP) has been known to transition to HA (Anada et al., 2008) and is further resorbed as HA.

Dicalcium phosphate (DCP) and dicalcium phosphate dihydrate (DCPD), commonly referred to as monetite and brushite respectively, are generally resorbed by direct chemical dissolution, and have some of the higher resorption rates among the calcium phosphates (Bohner et al., 2012). It has been found that DCP has a higher resorption rate *in vivo* when compared to DCPD, as hydroxyapatite is formed and precipitated during the degradation process for DCPD (Gbureck et al., 2007). These materials by themselves are brittle, with mechanical properties below the required limits for bone augmentation (Alge et al., 2012). To mitigate the fast resorption rate of this class of calcium phosphates, research groups have used DCPD as a cement, reinforced with a polymer, for example poly(propylene fumarate) (Alge et al., 2012), to increase mechanical properties, while maintaining biocompatibility and osteointegration capabilities.

Tricalcium phosphate (TCP) is generally produced in three allotropic forms, β -TCP, α -TCP, and α' -TCP, differing in crystalline structure and mechanical properties (D. Liu et al., 2013). The β -TCP formulation is appropriate for bone scaffolding due to the biological response and mechanical properties of this allotropic phase, with care given to avoid the α -phase during sintering or material preparation (D. Liu et al., 2013). β -TCP has been used as granules, as an injectable paste (Matsuno et al., 2008), or mixed with HA as it has proven osteoconductive and resorbable properties (Vorndran et al., 2008). Products such as the Integra MozaikStrip™ porous scaffolds (Integra, 80% TCP, 20% Type I collagen) and Integra MozaikPutty™ (Integra, 80% TCP, 20% Type I collagen) are commercially available for repairing small bone defects and voids. Ongoing research focuses on manufacturing methodologies that would enable TCP to be used in load bearing applications (Vorndran et al., 2008) as well as on biological augmentation using embedded biomolecules or cells to improve osteointegration (Kim et al., 2014). TCP is a very popular bioceramic that promotes long-term osteointegration at a rate similar to allografts (Kim et al., 2014). Concerns have been raised over products such as GeneX® paste composed of calcium sulfate and β -TCP due to inflammation response (Friesenbichler et al., 2014).

For calcium polyphosphate (CPP), the decreased Ca:P ratio allows the molecules to organize in a chain-like configuration, similar to a polymer, where the oxygen-bridged links can form linear structures (polyphosphates), ring structures (metaphosphates), or cage structures (ultraphosphates) with a random distribution in an amorphous state or in an organized distribution in a crystalline state (Pilliar et al., 2001). CPP can be sintered at different temperatures to produce a range of crystalline phases, with decreasing rates of degradation in order of CPP > α -CPP > β -CPP > γ -CPP (Qiu et al., 2006). CPP has been proven to be osteoconductive and osteoinductive *in vivo* (Kandel et al., 2006; Pilliar et al., 2007; Shanjani et al., 2013) via release of calcium and phosphate ions, with the main resorption mechanism being cell-mediated.

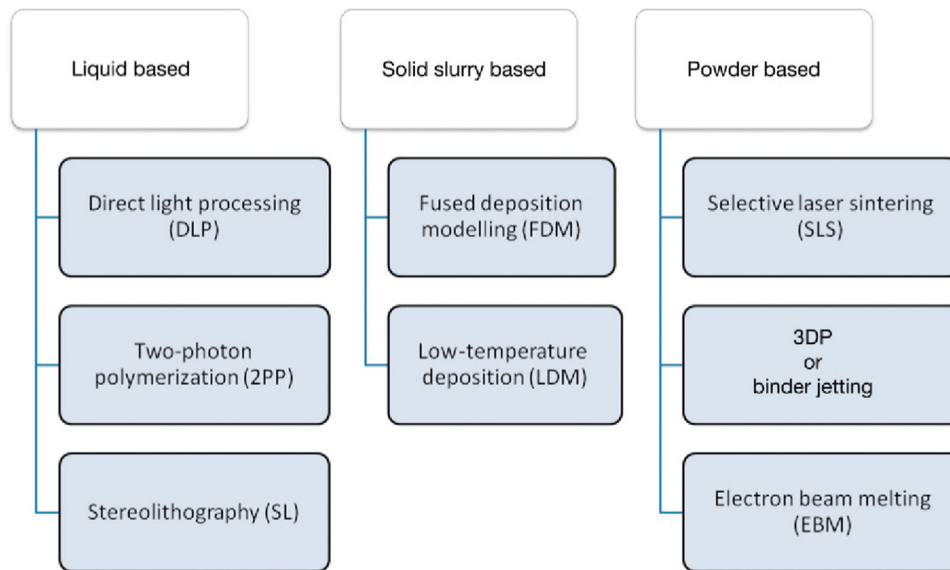
Aside from bioceramics, amorphous glasses, such as the bioactive glass, form a family of glass composites used in biomedical applications for bone augmentation or replacement (Rahaman et al., 2011). In spite of their inherent brittleness, bioactive amorphous glass materials have been extensively investigated, specifically the commercially available Bioglass® (45S5 glass) (Rahaman et al., 2011; Gerhardt and Boccaccini, 2010) due to their proven osteoconductivity and bonding to bone, as the material forms a HA-like layer once implanted at the site, which encourages bonds with the surrounding bone (Rahaman et al., 2011). Bioglass® is biocompatible; however, it has a limited resorption rate,

making it difficult to tune the resorption rate with the rate of new bone formation (Bohner, 2010; Rahaman et al., 2011). Borate bioactive glasses are also biocompatible and show faster degradation rates; however, there are some concerns over the toxicity of the borate ions *in vitro* and *in vivo* (Rahaman et al., 2011). Phosphate bioactive glasses offer the possibility of tuning the resorption rate by changing the composition (Rahaman et al., 2011). Bioactive glasses generally have an inherent brittleness (Rahaman et al., 2011) and it is also difficult to form them into porous 3D bone substitute structures or scaffolds (Rahaman et al., 2011; Gerhardt and Boccaccini, 2010).

There is a general trend toward producing composites of bioceramic or bioactive glass mixed with natural or synthetic polymers in order to meet the complex mechanical requirements in terms of compressive, tensile, shear, and fatigue properties for load bearing applications, as well as maintaining the biocompatible and bioresorbable characteristic of the bone substitute. Natural polymers such as chitosan, agarose, or collagen as well as synthetic polymers such as poly(ϵ -caprolactone) (PCL), poly(lactide-co-glycolide) (PLGA), poly(L-lactic acid) (PLLA), poly(L-lactide) (PLL), and poly(propylene fumarate) (PPF) are commonly used. The diversity of research done on composites, such as HA/PCL (Dorj et al., 2013), HA/PLGA (Kim et al., 2006), HA/PLLA (Wei and Ma, 2004), HA/ β -TCP/collagen (Maté-Sánchez de Val et al., 2014), HA/ β -TCP/agarose (Sánchez-Salcedo et al., 2008), HA/chitosan (Zhang et al., 2003), β -TCP/PLLA (D. Liu et al., 2013), β -TCP/chitosan (Dessi et al., 2013), bioglass/PLL (Zhang et al., 2004), and many more, are popular and can be used to control the resorption rate and mechanical properties of the material system. The prolific work done in this field may suggest that the optimal bone substitute scaffold material, structure, and properties have not yet been achieved. There are still limitations in achieving an acceptable range in mechanical and biochemical properties for resorbable ceramic and ceramic composites (Bohner et al., 2012). The key to solving this issue lies not only in considering the material itself, but also in implementing the appropriate complex 3D internal architecture of the construct from a structural and morphological point of view (Bohner et al., 2012; Butscher et al., 2011).

13.5.2 AM OF BIOCERAMIC MATERIALS: SEVERAL TECHNIQUES, LIMITATIONS, AND OPPORTUNITIES

Materials with controlled porous internal properties and complex 3D external characteristics are referred to as designer structures (Hollister, 2005). AM approaches are being refined toward achieving the goal of fabricating such designer structures in the context of producing bone substitutes. AM methodologies allow for parts to be built incrementally, layer-by-layer, based on information provided from a computer-aided design (CAD) program (Leong et al., 2003; Hutmacher et al., 2004). AM technologies strive to have each layer built to have the specific morphological configuration that would result in the desired micro- and macrostructure of the final part. In essence, AM offers the possibility of automated manufacturing of highly reproducible custom-shaped parts with controlled internal structure and custom external 3D architecture (Hutmacher et al., 2004). There are various AM techniques that can be used to construct bioceramic bone substitutes, depending on the type of raw materials used. Based on the ASTM F2792-12a standard terminology for AM technologies (ASTM International 2012), the general categories of AM methodologies are: (i) liquid-based methods such as direct light processing (DLP) and stereolithography (SL); (ii) solid or slurry extrusion-based methods such as fused deposition modeling (FDM) and low temperature deposition modeling (LDM); and (iii) powder-based methods such as SLS, DMLS, EBM, 3DP. These methods are summarized in Figure 13.5.

**FIGURE 13.5**

Classification of additive manufacturing methods for fabricating porous bioceramic bone substitutes.

13.5.2.1 Liquid-based AM Approaches

One class of AM systems relies on photopolymerization of liquid-based materials. In this AM fabrication approach, radiant energy is used to excite and initiate the polymerization process in low molecular weight monomers that are capable of forming long chain polymers (Yang et al., 2002). The family of polymers is called photopolymers. Biophotopolymers are a type of photopolymers that can biologically degrade by hydrolytic cleavage (Liska et al., 2007). A photopolymerization approach is DLP, where an array of micro-mirrors is used to direct light onto a photosensitive resin only at specific locations within each layer (Woesz et al., 2005; Skoog et al., 2014). SL is another photopolymerization method, where a UV laser moves in the x - y plane to irradiate and solidify a layer of UV curable polymer at specific locations (Lee et al., 2008; Skoog et al., 2014). The most common polymers used in SL for load bearing applications are poly(propylene fumarate) (PPF) (typically used with a diethylfumarate (DEF) cross-linking agent) and poly(D, L-lactide) (PDLLA), as they are biocompatible and bioresorbable, with attractive mechanical properties after cross-linking, specifically in producing bone substitutes for load bearing applications (Skoog et al., 2014). Skoog et al. (Skoog et al., 2014) have provided an excellent overview of the current state of the art on SL in tissue engineering, specifically for bone substitutes. They have outlined the benefits of SL in having a high degree of control over the internal and external architecture of the scaffolds, as well as the possibility of integrating bioactive ceramics fillers into the polymer matrix to enhance the mechanical and biological properties of the composite, at the cost of increasing the difficulty of SL processing, as the resulting resins are more viscous and more difficult to work with. For instance, HA powder was used with PPF/DEF (Lee et al., 2009) to produce intricate scaffolds with increased bioactivity. The most common use of SL in fabricating bone substitutes via indirect fabrication, where a mold is produced via SL and then

infiltrated with bioceramic slurries such as β -TCP (X. Li et al., 2007) or HA/ β -TCP (Sánchez-Salcedo et al., 2008) followed by a postprocessing protocol intended to remove the intermediate mold and to cure the bioceramics (Skoog et al., 2014). In general, photopolymerization is one of the most accurate AM techniques, with a high resolution, but it is limited to using photopolymeric materials (Stevens et al., 2008) and is generally used to manufacture bioceramic bone substitutes via indirect manufacturing by producing a mold.

13.5.2.2 Solid- or Slurry-based AM Approaches

Solid- or slurry-based AM methodologies rely on extrusion of molten materials or solids suspended in slurries. The most common method is FDM. A typical FDM machine deposits molten materials extruded through a heated nozzle displaced in the x – y plane to produce one layer following a specific geometric path based on CAD data. The layer is then displaced in the z -direction and a new layer can be built sequentially (Hutmacher et al., 2004). LDM and robocasting are methods that have a non heating liquefying deposition head to deposit a slurry that solidifies due to a low-temperature manufacturing environment (Xiong et al., 2002) or due to chemical setting (Dorj et al., 2013), respectively. Dorj et al. (Dorj et al., 2013) used a robocasting technique to prepare PCL/HA composites with increased mechanical properties by incorporating positively charged carbon nanotubes into the slurry-producing samples with a pore size of $\sim 230\text{ }\mu\text{m}$, with a compressive strength of up to 50 MPa, showing a considerable increase in mechanical properties with the addition of the carbon nanotubes. Liu et al. (Liu et al., 2009) used a PLGA/TCP slurry, while Xiong et al. (Xiong et al., 2002) employed PLLA/TCP slurry in the LDM process to produce bone substitutes with promising biological and mechanical properties. The most important benefit of these methods is the fact that there is a low risk of residual build materials being trapped inside the part. There is, however, a limited selection of materials that can be used for fabrication using these approaches (Hutmacher et al., 2004). These techniques also require the integration of extra support structures for building complex shapes with overhanging features. Using these methods, the nozzle dimension is one of the key limiting parameters that influence the feature size, as well as in dictating the speed of fabrication of the components.

13.5.2.3 Powder-based AM Approaches

Another class of AM techniques applies to powder-based materials. One such method is SLS, where a laser beam, typically a CO_2 laser, is used to locally raise the temperature of composite powders up to the glass transition temperature, resulting in fusion of neighboring particles and previous deposition layer (Yang et al., 2002). The powder can be a polymer, ceramic, metal, or composites (Leong et al., 2003). The laser is displaced in the x – y plane to create a layer based on CAD slice data. A roller mechanism spreads a new layer of powder and the process is repeated. The benefit of SLS is that there is generally no need for postprocessing steps. The process also allows for a wide variety of materials to be used.

For load bearing applications, researchers have looked at ways of using SLS to produce bioceramic or bioactive glass bone substitutes with appropriate mechanical and structural properties. For bioceramics, the task at hand is to enable the laser beam to bring the exposed powder ceramic material up to the sintering temperature to fuse particles together, without creating undesirable crystalline phases or inducing cracks in the part. Liu et al. (D. Liu et al., 2013) have investigated the appropriate material composition of a β -TCP powder blended with small amounts of PLLA (0.5–3 wt%) that would induce a liquid phase during sintering to decrease the sintering temperature of β -TCP, in order to avoid α -TCP phase transitions, achieving an increase of 18.8% in fracture toughness ($1.43 \pm 0.02\text{ MPa m}^{1/2}$) and 4.5% in compressive strength ($17.67 \pm 0.04\text{ MPa}$) when

compared to the pure sintered β -TCP. Shuai et al. have also looked at improving the mechanical properties of pure β -TCP constructs by changing the SLS fabrication parameters, resulting in 3.59 GPa hardness and $1.16 \text{ MPa m}^{1/2}$ fracture toughness (Shuai et al., 2013). SLS fabrication using bioactive glasses typically results in weaker constructs, intended for maxillofacial applications, such as Bioglass 58S and poly (D, L) lactide (PDLLA) scaffolds with a maximum reported compressive strength of 1.68 MPa (Pereira et al., 2014). Similarly, Bioglass[®] 45S5 bone substitutes have also been investigated using SLS, with special consideration given to phase transitions in the glass material during sintering, targeting the $\text{Na}_2\text{Ca}_2\text{Si}_3\text{O}_9$ final phase (J. Liu et al., 2013) and resulting in a maximum fracture toughness of $0.57 \text{ MPa m}^{1/2}$.

The major disadvantage of SLS techniques is that it is difficult to remove the powder trapped within the parts, limiting the overall feature size that can be achieved using this method (Leong et al., 2003). Also, because of the high temperature involved during the manufacturing process, the materials may thermally degrade or produce undesirable phases, reducing the mechanical strength or biological compatibility of the part (Leong et al., 2003; D. Liu et al., 2013). High temperatures will also lead to unwanted bonding among adjoining powder particles, decreasing resolution, accuracy, and repeatability, and making it difficult to build features less than $400 \text{ }\mu\text{m}$ (Yang et al., 2002). The feature size is limited by the laser spot size, and generally the SLS methodology suffers from slow fabrication rates. In addition, SLS systems are inherently expensive as they employ a laser system.

3DP, also known as adhesion bonding or binder jetting, is another category of powder-based AM methodologies (Yang et al., 2002). 3DP allows parts to be manufactured in a layer-by-layer fashion based on information provided by a software program. A 3D model is first designed in CAD software and then sectioned into bottom-up images corresponding to parallel slices or layers. 3DP employs powders as the substrate material for constructing the designed parts in a layer-by-layer fashion (Butscher et al., 2011). Within each layer, the powder particles are first spread onto a building area using a counter-rotating roller and then glued together at locations corresponding to the slice layer image by injecting a binder using an ink-jet printhead or similar printing technology (Cima et al., 1993, 1994, 1995). The building area moves down, allowing for new layers of powder to be spread sequentially until the part is completed. The resulting product is referred to as the green part. Further postprocessing is typically required to fully cure or anneal the green parts (Hutmacher, 2000; Yang et al., 2002).

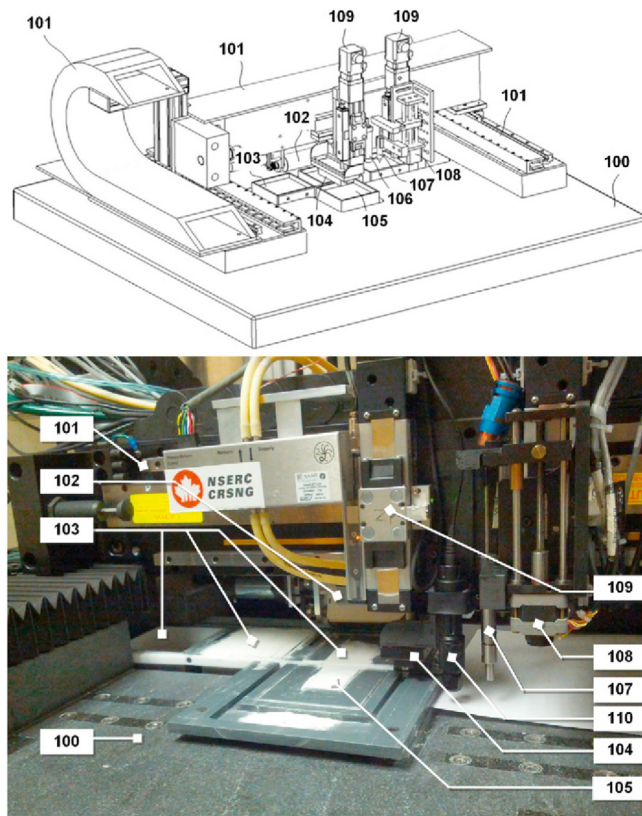
3DP is a very popular methodology in manufacturing bone substitutes using bioceramic and bioglass powders. Typically, in this methodology, the bioceramic material is either coated or mixed with another polymer that can dissolve when exposed to the printed liquid binder and act as a glue to enhance the green part strength. Calcium phosphates have been widely used in 3DP by different groups as the powder material, where researchers have used CPP blended with a small wt% of PVA powder (Shanjani et al., 2011), where in a subsequent study Hu et al. (Hu et al., 2014) reported an increase in compressive strength from $21 \pm 4.5 \text{ MPa}$ for conventionally fabricated CPP/PVA samples to $50.2 \pm 4.74 \text{ MPa}$ for 3DP CPP/PVA samples, with good osteointegration *in vivo* (Shanjani et al., 2013). Instead of bioceramic/polymer powder blending, other research groups have used polymer coatings of ceramic powders such as HA with a maltodextrin coating (Chumnanklang et al., 2007), resulting in porosity ranges between 59–65% and an upper limit on flexural strength of approximately 1.5 MPa. Another approach is to set the bioceramic material into a cement by jetting a reagent, for example phosphoric acid to cement TCP (Gbureck et al., 2008, 2007, 2007), with reported compressive strength of up to $21.7 \pm 1.1 \text{ MPa}$, porosity of 38–44%, and good osteointegration (Habibovic et al., 2008).

A significant benefit of the 3DP methodology is that a wide variety of materials in powder form such as polymers, ceramics, metals, and composites can be used to manufacture products (Butscher et al., 2011; Stevens et al., 2008; Yang et al., 2002; Castilho et al., 2011). Other key advantages include: ease of customizing, scaling, and reproducing complex-shaped designs with no need for support structures (Stevens et al., 2008; Hutmacher et al., 2004; Leong et al., 2003; Yang et al., 2002); green part manufacturing at room temperature (Hutmacher et al., 2004; Leong et al., 2003; Yang et al., 2002; Castilho et al., 2013, 2011); and digital control of 3D shape design and macro features such as embedded interconnected channels (Hutmacher, 2000; Stevens et al., 2008; Hollister, 2005; Hutmacher et al., 2004; Castilho et al., 2013). For some applications, such as tissue scaffold design, the inherent microporosity resulting from the arrangement of powder particles in the AM process is also a highly beneficial feature (Hollister, 2005; Hutmacher et al., 2004; Leong et al., 2003).

One of the most pressing drawbacks is that the smallest feature size of conventional powder-based 3DP techniques is limited by the binder droplet volume (Butscher et al., 2011), powder particle size with appropriate flowability (Butscher et al., 2011; Leong et al., 2003; Yang et al., 2001), powder compaction force (Shanjani, 2011), and the high potential for having trapped particles inside cavities formed within the part (Butscher et al., 2011; Hutmacher et al., 2004; Leong et al., 2003; Yang et al., 2002). Overall, it is difficult to achieve features below 500 μm in size using this fabrication method (Butscher et al., 2011; Hutmacher et al., 2004; Leong et al., 2003; Yang et al., 2002; Castilho et al., 2011). This issue becomes even more pressing in manufacturing constructs with complex conformal channels, as it becomes increasingly difficult to remove trapped support materials from features within the parts. Other emerging limiting aspects of conventional powder-based 3DP techniques are the use of only one powder size or powder type during manufacturing, the application of a constant compaction force during powder layer spreading, the utilization of a single layer thickness setting throughout the part, and the lack of control over the gray-scale gradient of binder volume dispersed within each layer. These aspects impose limitations in manufacturing of porous scaffolds with heterogeneous or functionally graded properties as required in various industrial and biomedical applications.

To address the current limitations in 3DP technology, a mechatronic system was designed by the authors to control the fabrication of functionally graded internal features, porosities, and material properties of parts. To this end, a variable porosity AM system via 3DP was developed as seen in Figure 13.6, where a collection of control modules were incorporated to achieve the required performance. Such controlled devices include a counter-rotating roller module, multiple supply bed selection and alignment module, sacrificial porogen particle insertion module, sacrificial polymer deposition module, UV curing module, and an environment control module. The sacrificial porogen particle insertion module and sacrificial polymer deposition module assemblies are used to produce a controlled porous feature size in the range of 100–500 μm , which can be achieved by selectively depositing either porogens or sacrificial polymeric structures on specific layers that can thermally disintegrate during postprocessing, leaving behind controlled porosities and/or interconnected channels. This approach allows for control of internal features by preventing loose support powder material from being trapped inside complex cavities of parts, with results available in literature (Vlasea et al., 2013; Vlasea and Toyserkani, 2013). The system has been tested in the context of fabricating porous bone substitutes using CPP bioceramic powder.

The newly developed multiscale 3DP system allows for the capability of selecting between three powder feed compartments during runtime to produce layers with different material composition at selected locations throughout the part. To the author's knowledge, such a system is not commercially available, nor is it discussed in the literature thus far. As a preliminary work presented as a case study here, to

**FIGURE 13.6**

Novel multiscale 3DP additive manufacturing system for fabrication of functionally graded porous structures. (100) Granite support, (101) Precision xy gantry assembly, (102) Counter rotating roller module, (103) Multiple supply bed selection and alignment module, (104) Binder dispensing print head module, (105) Build bed module, (106) Sacrificial porogen particle insertion module, (107) Microsyringe sacrificial polymer deposition module, (108) Microsyringe deposition control, (109) Precision control z-axis, (110) UV curing module.

evaluate the performance of the multipowder system configuration, two powder particle sizes were selected, CPP bioceramic powder with 75–150 μm particle size (hereafter referred to as large), and <75 μm particle size (hereafter referred to as small), with a morphology and particle distribution shown in [Figure 13.7](#). Three categories of parts were manufactured, with small, large, and dual (50/50) powder composition, respectively. The large CPP and small CPP powders were blended, respectively, with 10% polyvinyl alcohol (PVA) powder (Alfa Aesar, Ward Hill, MA) and with particle size <63 μm . The fabricated test parts were 4 mm in diameter and 6 mm tall, printed with a layer thickness of 150 μm . The green parts were then sintered in a high-temperature furnace (Lindberg/Blue M, ThermoScientific) with an established heat treatment protocol ([Pilliar et al., 2009](#)).

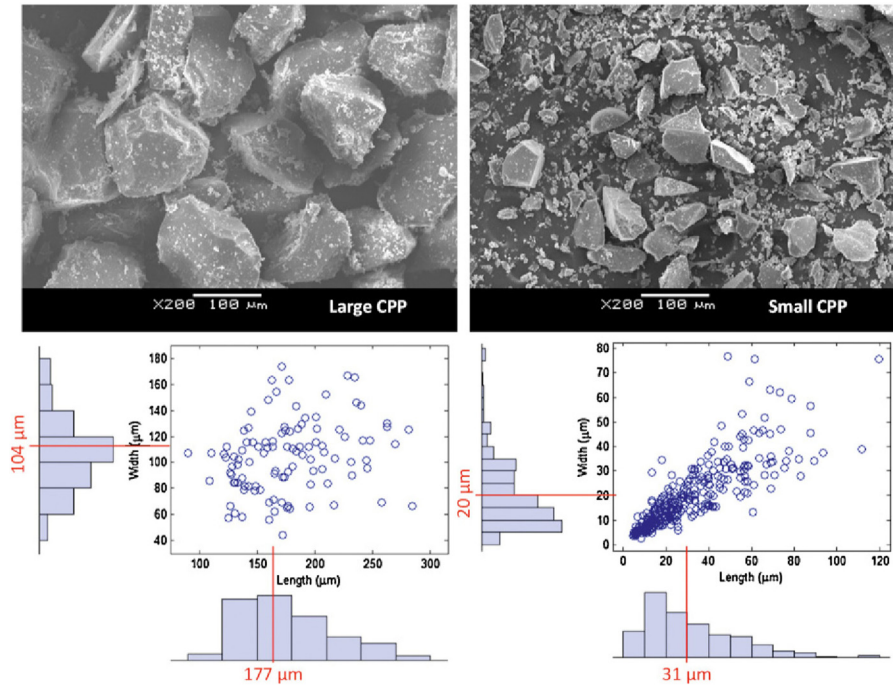
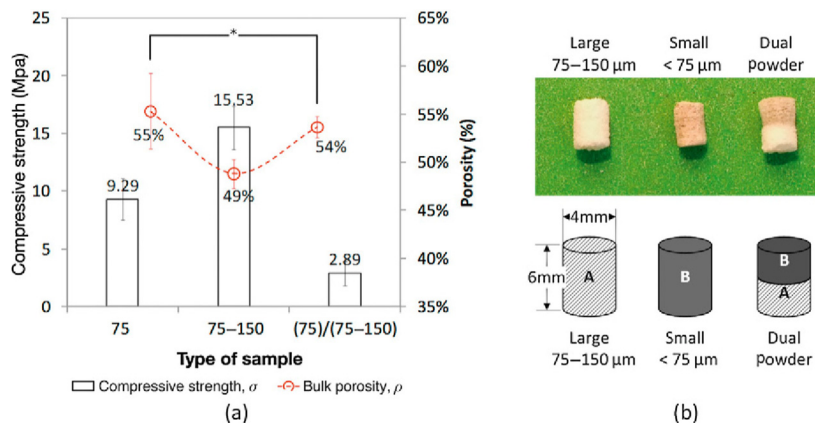


FIGURE 13.7

The particle morphology and corresponding size distribution for large particle size, sieved between 75–150 μm ($n = 105$) and small particle size, sieved at < 75 μm, ($n = 300$).

Figure 13.8a illustrates the compressive strength measurements plotted against porosity values, with statistical significance derived from one-way ANOVA. The mechanical compression test results were surprising. The category of samples with the highest compressive strength corresponded to parts manufactured using the large powder size, with a compressive strength of 15.5 ± 1.9 MPa, the result being corroborated by bulk density and bulk porosity measurements.

The literature suggests that samples with a lower particle size composition manufactured using 3DP approaches would result in parts with a higher mechanical compressive strength (Will et al., 2008). The discrepancy between expected results and the actual results obtained in this chapter can be explained by looking at powder flowability, layer thickness, binder imbibition, and most noteworthy, the sintering protocol as a function of powder particle size. Small particles tend to have poor flowability (Butscher et al., 2011) and are inclined to agglomerate. This effect causes limited powder compaction during layer spreading (Shanjani et al., 2011) and may explain the poor mechanical strength of the samples manufactured using powder with small particle size in this chapter. The parts with the lowest performance were the dual powder composition samples. This may have been caused by stresses occurring at the interface between the two powder types during sintering, as indicated by the fracture patterns occurring at that location. Another aspect that influences part strength is the sintering process. In this work, all samples were subjected to the same sintering protocol, optimized for the 75–150 μm powder particle range. Figure 13.8b

**FIGURE 13.8**

(a) Bulk porosity characteristics and compressive strength measurements of cylindrical samples with small, large, and dual particle size composition, with ($p < 0.05$). For each trial, ($n = 10$). (*) Illustrates Tukey HSD statistical pairwise similarity. (b) Sintered samples with large, small, and dual powder composition.

illustrates examples of sintered CPP samples corresponding to the three categories of manufactured parts. It can be seen that the parts have a significantly different color, indicating that sintering had a different effect on samples, depending on powder size composition. A more in-depth investigation into an appropriate sintering protocol for samples with different powder size layer composition should be investigated.

The important achievement of this work is in demonstrating the feasibility of using multiple powders during the multiscale 3DP manufacturing process, which advances the current state of powder-based AM toward new manufacturing opportunities. Further investigations into increasing the performance of this approach are currently underway.

13.6 NANOCOMPOSITE BONE SUBSTITUTES

13.6.1 NANOMATERIALS, LIMITATIONS, AND OPPORTUNITIES

Bone is inherently a nanocomposite, which serves as a mechanical support to the body and also a reservoir for essential minerals (James et al., 2011; Murugan and Ramakrishna, 2005). There have been many studies on the development of synthetic bone substitutes such as ceramic-based and polymer-based materials. In addition to certain requirements of these materials like high mechanical strength, biodegradability, and biocompatibility, they should be shaped into 3D porous structures to fit into the defected part of the bone. Osteoconductivity of the scaffolds is of great importance since it determines the rate of osteoblast cell infiltration and proliferation. The scaffolds should also be degraded with a similar rate to the bone healing process and the yield of degradation must not provoke immune responses in the body. Commonly, all these requirements are not obtained through a single-phase material and there is a significant need for the development of multiphase materials (i.e. composites with similar physical and structural properties to bone). Bone is composed of a nanocomposite known as

ECM which consists of an organic matrix, osteoid, and an inorganic phase as reinforcement. Fibrous protein and collagen are the main components of the organic matrix, whereas the inorganic phase is mainly made of carbonate-substituted hydroxyapatite (HA) crystals. The mineral is not pure HA and some traces of magnesium, carbonate, and zinc elements are mostly detected in the structure. In addition, calcium-to-phosphorous ratio is not constant and might undergo significant variations at the same bone site, but in different populations or age groups (Rogel et al., 2008; Crystals et al. 2006).

For bone grafting, ceramic materials, such as calcium phosphates and hydroxyapatite, are conventionally employed as fillers and coatings. Although they provide good osteoconductivity, their scaffolds are brittle and undergo detrimental failure under load. They also suffer from slow biodegradability, which impedes an efficient bone healing process. Polymeric materials have been investigated widely as bone grafts due to their high biocompatibility and proper mechanical strength. Polylactic acid (PLA), polyglycolic acid (PGA), poly(ϵ -caprolactone) (PCL), and their copolymers are the most common polymers used for tissue engineering. It has been reported that ceramic/polymer composites represent improved biological and mechanical properties. Incorporation of synthetic nanocomposites along with a proper micro- and macrostructural design can further enhance the performance of bone grafts. Higher surface area and higher grain boundaries can improve the toughness and mechanical properties of the structures significantly. Additionally, nanocomposites provide enhanced osteoblast cell adhesion and proliferation. There are many reports on the development of ceramic, polymeric, and metallic nanocomposites for load-bearing applications. However, manufacturing of porous structures with proper interconnectivity and strength is still challenging and needs more in-depth investigation.

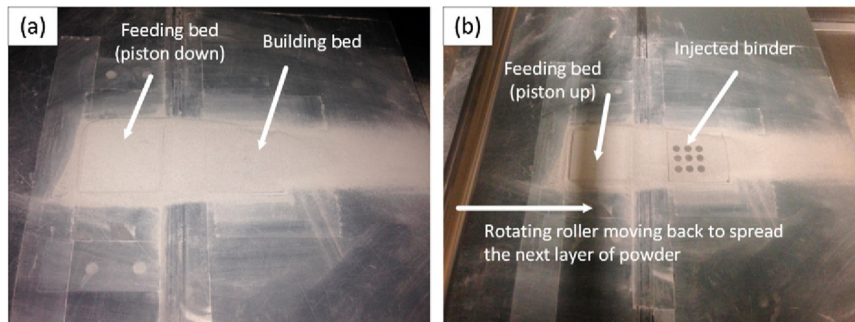
13.6.2 AM OF NANOCOMPOSITES: SEVERAL TECHNIQUES, LIMITATIONS AND OPPORTUNITIES

Zhou et al. (Zhou et al. 2008) reported the synthesis of PLLA/HA nanocomposite with the HA particle size of around 20 nm. The scaffolds were built using SLS at the layer thickness of 100 μm . It was shown that using inappropriate laser power, part bed temperature (PBT) and scan spacing (SS) can lower the resolution and quality of 3D printed structures. *In vitro* cell culture experiments indicated promising results in regards to osteoconductivity and proliferation of osteoblast cells.

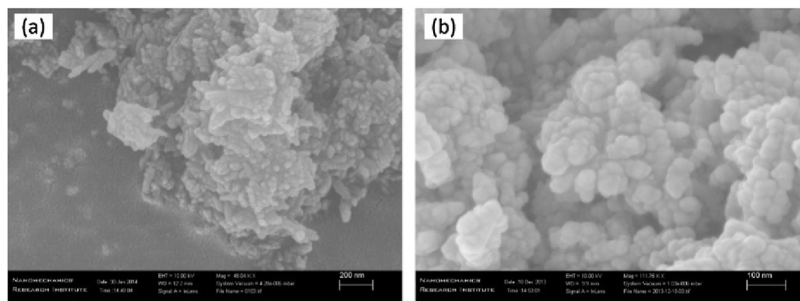
Heo et al. (Heo et al., 2009) studied layer-by-layer manufacturing of HA/PCL composite fabricated with nano (n-HPC) and micro HA (m-HPC) particles, respectively, prepared through a solvent casting method. 3D-printed scaffolds were freeze-dried after immersion in distilled water. The SEM images of the n-HCP and m-HCP scaffolds reveal that the n-HCP scaffolds have a smooth surface while that of m-HCP is rough with less consistency. It was also shown that the attachment of cells, alkaline phosphatase activity, calcium content, and mechanical strength were higher in n-HPC compared to that of m-HPC scaffolds.

In another study, calcium phosphate /poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) and HA/PLLA nanocomposites were synthesized and 3D-printed into scaffolds using SLS (Duan and Wang 2010a, 2010b; Duan et al., 2010, 2011). Osteoconductivity and biodegradability are provided by calcium phosphate and polymer matrix, respectively. Proximal femoral condyle sintered scaffolds show high quality prints compared to the model. The mechanical properties of the nanocomposite scaffolds were shown to be higher than their polymeric counterparts in dry state. It was also reported that SaOS-2 osteoblast cell adhesion, proliferation, and ALP activity of the structures were significantly improved using the nanocomposite.

Poly-lactide-co-glycolide acid (PLGA) and nanotitania particles are also known as biocompatible materials. Liu et al. (Liu and Webster, 2011) fabricated titania/PLGA nanocomposite dispersion

**FIGURE 13.9**

Fabrication process of 3D printed structures by powder-bed AM, (a) feeding bed and building bed are indicated, (b) the compartment moves forward to inject the binder on the building bed, one layer of binder is injected on the powder bed, compartment moves backward to spread another layer of powder onto the building bed.

**FIGURE 13.10**

SEM images of HG4 nanocomposite, (a) graphene-coated Hap particles; (b) showing higher magnifications. The nanoparticles seen in (b) could be attributed to agglomerated graphene oxide sheets.

via a solvent casting technique and used aerosol-based M3D™ 3D printing system (M3D™ developed by OPTOMECH) to produce 3D scaffolds of titania/PLGA nanocomposite. The results indicated better mechanical properties and biological performance than PLGA control samples.

Graphene has been reported to improve mechanical properties and osteoblast cell adhesion and proliferation significantly (Zhang et al., 2013; Zanin et al., 2013; Marques et al., 2012; Biris et al., 2011; Ma et al., 2012; Liu et al., 2012; Rodríguez-Lorenzo, 2009). Azhari et al. (Azhari et al., 2014) investigated the AM of graphene/hydroxyapatite nanocomposite using layer-by-layer 3D-printing technique. As illustrated in Figure 13.9, the rotating roller spread a layer of powder onto the building bed where an aqueous binder is injected to adhere particles based on the sliced CAD model. The process is iterated until the object is built up. Graphene oxide was mixed with HA particles through a wet mixing process to fabricate HA/graphene nanocomposite. SEM images of the nanocomposite powder show that HA flakes are fully decorated by graphene particles (see Figure 13.10). This texture can lower the van der Waals attractive forces between the particles. It was shown that the flowability of the nanocomposite powder, which is an

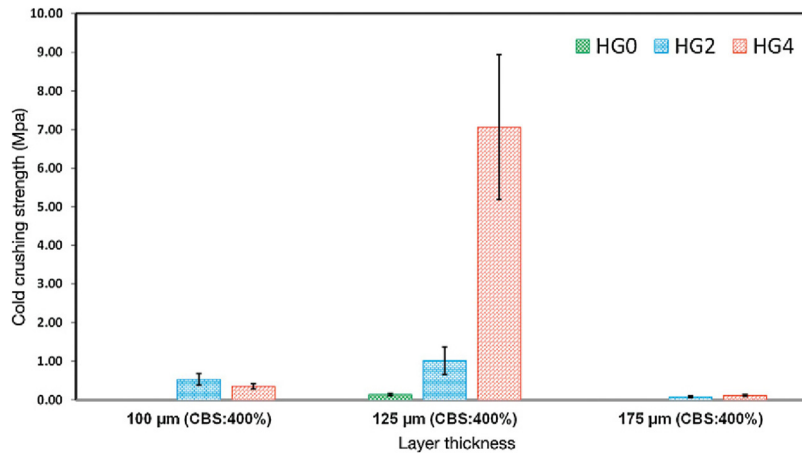


FIGURE 13.11

Cold crushing strength of HG0, HG2, and HG4 specimens 3D-printed at layer thicknesses of 100, 125, and 175 μm with SBS/CBS ratio of 100/400%.

essence in the manufacturing process, was improved significantly by the addition of graphene. The samples printed at the layer thickness of 125 μm with the shell binder saturation (SBS) to core binder saturation level (CBS) of 100/400% showed the highest mechanical strength. As seen in Figure 13.11, the compressive strength of the 3D-printed cylinders was increased from ~ 0.1 to ~ 7.0 MPa in the samples with the graphene content of only 0.4 wt%.

In summary, it was realized that the flowability of HA particles decorated with graphene was improved significantly. This phenomenon will enhance the resolution and quality of the structures 3D-printed through AM. It was also shown that the mechanical strength of HG4 green specimens was almost 70 times more than HG0 specimens. In future efforts to employ the samples for bioimplantation, the mechanical properties and biocompatibility of the sintered samples should be investigated thoroughly.

13.7 Conclusions

Metals still form the bulk of primary bone substitute materials in the orthopedic industry. Interest in metal implants will remain strong in the future, especially for senior patients. The fabrication of metal implants is costly, but with new developments in the AM field, manufacturability may become easier. Attention has been focused on the use of bioceramics in the fabrication of bone substitutes due to their biochemical properties. To improve on the mechanical properties of bioceramics, polymer matrix composites are typically used in various AM approaches. Owing to the high degree of similarity between bone microstructure as a nanocomposite, biocompatible nanocomposites are also arousing interest in bone regenerative medicine and have been employed using AM approaches. Overall, AM methodologies offer the possibility of producing complex bone substitute implants, specifically for load bearing applications, with a complex 3D external shape, and intricate

porous internal architecture. The extensive ongoing research in the field of materials and manufacturing methodologies suggests that there are still barriers that need to be overcome to achieve consistently reliable and safe substitutes in regenerating or augmenting bone function.

References

- Alge, D.L. et al., 2012. Poly(propylene fumarate) reinforced dicalcium phosphate dihydrate cement composites for bone tissue engineering. *Journal of biomedical materials research. Part A*, 100(7), pp. 1792–802. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22489012>. [Accessed June 15, 2012].
- Alvarez, K. & Nakajima, H., 2009. Metallic Scaffolds for Bone Regeneration. *Materials*, 2(3), pp. 790–832. Available at: <http://www.mdpi.com/1996-1944/2/3/790/>. [Accessed May 27, 2014].
- Anada, T. et al., 2008. Dose-Dependent Osteogenic Effect of Octacalcium Phosphate on Mouse Bone Marrow Stromal Cells. *Tissue Engineering Part A*, 14(6), pp. 965–978. Available at: <http://www.liebertonline.com/doi/abs/10.1089/ten.tea.2007.0339>. [Accessed June 15, 2014].
- ASTM International, 2012. *ASTM F2792-12a Standard Terminology for Additive Manufacturing Technologies*. Available at: <http://www.astm.org/Standards/F2792.htm>.
- Azhari, A. et al., 2014. Additive Manufacturing of Graphene–Hydroxyapatite Nanocomposite Structures. *International Journal of Applied Ceramic Technology*, pp. 1–10. Available at: <http://onlinelibrary.wiley.com/doi/10.1111/ijac.12309/full>.
- Balla, V.K. et al., 2010. Porous tantalum structures for bone implants: fabrication, mechanical and in vitro biological properties. *Acta biomaterialia*, 6(8), pp. 3349–59. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2883027&tool=pmcentrez&rendertype=abstract>. [Accessed June 20, 2014].
- Bartel, D. L., Davy, D. T., & Keaveny, T. M. (2006). *Orthopaedic biomechanics: mechanics and design in musculoskeletal systems First*. Upper Saddle River: Pearson Prentice Hall.
- Basalah, A. et al., 2012. Characterizations of additive manufactured porous titanium implants. *Journal of biomedical materials research. Part B, Applied biomaterials*, 100(7), pp. 1970–9. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22865677>. [Accessed June 20, 2014].
- Billi, F. & Campbell, P., 2010. Nanotoxicology of metal wear particles in total joint arthroplasty: a review of current concepts. *Journal of applied biomaterials & biomechanics: JABB*, 8(1), pp. 1–6. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20740415>.
- Biris, A.R. et al., 2011. Novel Multicomponent and Biocompatible Nanocomposite Materials Based on Few-Layer Graphenes Synthesized on a Gold/Hydroxyapatite Catalytic System with Applications in Bone Regeneration. *The Journal of Physical Chemistry C*, 115(39), pp. 18967–18976. Available at: <http://pubs.acs.org/doi/abs/10.1021/jp203474y>.
- Bohner, M. et al., 2011. Commentary: Deciphering the link between architecture and biological response of a bone graft substitute. *Acta biomaterialia*, 7(2), pp. 478–84. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20709195>. [Accessed November 15, 2012].
- Bohner, M., 2010. Resorbable biomaterials as bone graft substitutes. *Materials Today*, 13(1–2), pp. 24–30. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S1369702110700146>. [Accessed May 1, 2014].
- Bohner, M., Galea, L. & Doebelin, N., 2012. Calcium phosphate bone graft substitutes: Failures and hopes. *Journal of the European Ceramic Society*, 32(11), pp. 2663–2671. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0955221912001021>. [Accessed October 26, 2012].
- Bose, S. & Tarafder, S., 2012. Calcium phosphate ceramic systems in growth factor and drug delivery for bone tissue engineering: A review. *Acta biomaterialia*, 8(4), pp. 1401–21. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22127225>. [Accessed March 16, 2012].

- Butscher, A. et al., 2011. Structural and material approaches to bone tissue engineering in powder-based three-dimensional printing. *Acta Biomaterialia*, 7(3), pp. 907–20. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20920616>. [Accessed March 8, 2013].
- Cai, S. & Xi, J., 2008. A control approach for pore size distribution in the bone scaffold based on the hexahedral mesh refinement. *Computer-Aided Design*, 40(10–11), pp. 1040–1050. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0010448508001838>. [Accessed May 21, 2014].
- Campanelli, S., et al. (1994). Capabilities and Performances of the Selective Laser Melting Process. In J. Meng (Ed.), *New Trends in Technologies: Devices, Computer* (pp. 233–254). Communication and Industrial Systems.
- Castilho, M. et al., 2013. Fabrication of computationally designed scaffolds by low temperature 3D printing. *Biofabrication*, 5(3), p.035012. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23887064>. [Accessed September 28, 2013].
- Castilho, M. et al., 2011. Structural evaluation of scaffolds prototypes produced by three-dimensional printing. *The International Journal of Advanced Manufacturing Technology*, 56(5–8), pp. 561–569. Available at: <http://link.springer.com/10.1007/s00170-011-3219-4>. [Accessed March 26, 2013].
- Chang, B.S. et al., 2000. Osteoconduction at porous hydroxyapatite with various pore configurations. *Biomaterials*, 21(12), pp. 1291–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19845154>.
- Cheng, S. et al., 2013. *Hip and knee replacements in Canada: Canadian joint replacement registry 2013 annual report*, Ottawa. Available at: https://secure.cihi.ca/free_products/CJRR_2013_Annual_Report_EN.pdf.
- Chumanklang, R. et al., 2007. 3D printing of hydroxyapatite: Effect of binder concentration in pre-coated particle on part strength. *Materials Science and Engineering: C*, 27(4), pp. 914–921. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S092849310600378X>. [Accessed August 22, 2013].
- Cima, M. et al., 1995. Three Dimensional Printing, Patent Number 5,387,380.
- Cima, M.J. et al., 1994. Three Dimensional Printing Techniques, Patent Number 5,340,656.
- Cima, M.J. et al., 1993. Three-dimensional printing techniques, Patent Number 5,204,055.
- Crystals, N.C.H. et al., 2006. Collagen Scaffolds Reinforced with Biomimetic Composite., 12.(9).
- Curodeau, a, Sachs, E. & Caldarise, S., 2000. Design and fabrication of cast orthopedic implants with freeform surface textures from 3-D printed ceramic shell. *Journal of biomedical materials research*, 53(5), pp. 525–35. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10984701>.
- Dabrowski, B. et al., 2010. Highly porous titanium scaffolds for orthopaedic applications. *Journal of biomedical materials research. Part B, Applied biomaterials*, 95(1), pp. 53–61. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20690174>. [Accessed June 20, 2014].
- Dessi, M. et al., 2013. Novel biomimetic thermosensitive β -tricalcium phosphate/chitosan-based hydrogels for bone tissue engineering. *Journal of biomedical materials research. Part A*, 101(10), pp. 2984–93. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23873836>. [Accessed June 15, 2014].
- Detsch, R. et al., 2010. The resorption of nanocrystalline calcium phosphates by osteoclast-like cells. *Acta biomaterialia*, 6(8), pp. 3223–33. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20206720>. [Accessed May 22, 2014].
- Dinda, G.P., Song, L. & Mazumder, J., 2008. Fabrication of Ti-6Al-4V Scaffolds by Direct Metal Deposition. *Metallurgical and Materials Transactions A*, 39(12), pp. 2914–2922. Available at: <http://link.springer.com/10.1007/s11661-008-9634-y>. [Accessed June 20, 2014].
- Dorj, B. et al., 2013. Robocasting nanocomposite scaffolds of poly(caprolactone)/hydroxyapatite incorporating modified carbon nanotubes for hard tissue reconstruction. *Journal of biomedical materials research. Part A*, 101(6), pp. 1670–81. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23184729>. [Accessed June 4, 2014].
- Duan, B., Wang, M., Li, Z.Y., et al., 2010. Surface modification of three-dimensional Ca-P/PHBV nanocomposite scaffolds by physical entrapment of gelatin and its in vitro biological evaluation. *Frontiers of Materials Science*, 5(1), pp. 57–68. Available at: <http://link.springer.com/10.1007/s11706-011-0101-0>. [Accessed June 12, 2014].

- Duan, B., Wang, M., Zhou, W.Y., et al., 2010. Three-dimensional nanocomposite scaffolds fabricated via selective laser sintering for bone tissue engineering. *Acta biomaterialia*, 6(12), pp. 4495–505. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20601244>. [Accessed June 12, 2014].
- Duan, B., Cheung, W.L. & Wang, M., 2011. Optimized fabrication of Ca-P/PHBV nanocomposite scaffolds via selective laser sintering for bone tissue engineering. *Biofabrication*, 3(1), p.015001. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21245522>. [Accessed June 12, 2014].
- Duan, B. & Wang, M., 2010a. Customized Ca-P/PHBV nanocomposite scaffolds for bone tissue engineering: design, fabrication, surface modification and sustained release of growth factor. *Journal of the Royal Society, Interface / the Royal Society*, 7 Suppl 5, pp.S615–29. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3024573&tool=pmcentrez&rendertype=abstract> [Accessed June 12, 2014].
- Duan, B. & Wang, M., 2010b. Encapsulation and release of biomolecules from Ca–P/PHBV nanocomposite microspheres and three-dimensional scaffolds fabricated by selective laser sintering. *Polymer Degradation and Stability*, 95(9), pp. 1655–1664. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0141391010002247>. [Accessed June 12, 2014].
- Friesenbichler, J. et al., 2014. Adverse reactions of artificial bone graft substitutes: lessons learned from using tricalcium phosphate geneX[®]. *Clinical orthopaedics and related research*, 472(3), pp. 976–82. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24078171>. [Accessed June 15, 2014].
- Frosch, K.-H., & Stürmer, K. M. (2006). Metallic Biomaterials in Skeletal Repair. *European Journal of Trauma*, 32(2), 149–159.
- Frost, H.M., 2003. Bone's mechanostat: a 2003 update. *The anatomical record. Part A, Discoveries in molecular, cellular, and evolutionary biology*, 275(2), pp. 1081–101. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/14613308>. [Accessed August 7, 2013].
- Gbureck, U., Hölzel, T., Doillon, C.J., et al., 2007. Direct printing of bioceramic implants with spatially localized angiogenic factors. *Advanced Materials*, 19(6), pp. 795–800. Available at: <http://doi.wiley.com/10.1002/adma.200601370>. [Accessed August 20, 2013].
- Gbureck, U. et al., 2008. Preparation of tricalcium phosphate/calcium pyrophosphate structures via rapid prototyping. *Journal of Materials Science: Materials in Medicine*, 19(4), pp. 1559–63. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/18236137>. [Accessed August 20, 2013].
- Gbureck, U., Hölzel, T., Klammert, U., et al., 2007. Resorbable Dicalcium Phosphate Bone Substitutes Prepared by 3D Powder Printing. *Advanced Functional Materials*, 17(18), pp. 3940–3945. Available at: <http://doi.wiley.com/10.1002/adfm.200700019>. [Accessed May 26, 2014].
- Gerhardt, L.-C. & Boccaccini, A.R., 2010. Bioactive Glass and Glass-Ceramic Scaffolds for Bone Tissue Engineering. *Materials*, 3(7), pp. 3867–3910. Available at: <http://www.mdpi.com/1996-1944/3/7/3867/>. [Accessed May 30, 2014].
- Gikas, P.D. et al., 2009. An overview of autologous chondrocyte implantation. *Bone and Joint Surgery - British Volume*, 91(8), pp. 997–1006. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19651824>. [Accessed July 29, 2010].
- Gryn timer, M.D. et al., 2002. Porous calcium polyphosphate scaffolds for bone substitute applications in vivo studies. *Biomaterials*, 23(9), pp. 2063–70. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11996048>.
- Habibovic, P. et al., 2008. Osteoconduction and osteoinduction of low-temperature 3D printed bioceramic implants. *Biomaterials*, 29(7), pp. 944–53. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/18055009>. [Accessed August 20, 2013].
- Heo, S. et al., 2009. In Vitro and Animal Study of Novel Nano-Scaffolds Fabricated by Layer Manufacturing Process., 15(5).
- Hollister, S.J., 2005. Porous scaffold design for tissue engineering. *Nature materials*, 4(7), pp. 518–24. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16003400>.
- Hu, Y. et al., 2014. Porous calcium polyphosphate bone substitutes: additive manufacturing versus conventional gravity sinter processing-effect on structure and mechanical properties. *Journal of biomedical*

- materials research. Part B, *Applied biomaterials*, 102(2), pp. 274–83. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23997039>. [Accessed April 14, 2014].
- Hutchinson, M., 2009. The burden of musculoskeletal diseases in the united states: prevalence, societal and economic cost. *Journal of the American College of Surgeons*, 208(1), pp.e5–e6. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S1072751508007357>.
- Hutmacher, D.W., 2000. Scaffolds in tissue engineering bone and cartilage. *Biomaterials*, 21(24), pp. 2529–43. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11071603>.
- Hutmacher, D.W., Sittering, M. & Risbud, M.V., 2004. Scaffold-based tissue engineering: rationale for computer-aided design and solid free-form fabrication systems. *Trends in Biotechnology*, 22(7), pp. 354–62. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15245908>. [Accessed July 29, 2010].
- James, R. et al., 2011. Nanocomposites and bone regeneration. *Frontiers of Materials Science*, 5(4), pp. 342–357. Available at: <http://link.springer.com/10.1007/s11706-011-0151-3>. [Accessed June 12, 2014].
- Kandel, R.A. et al., 2006. Repair of osteochondral defects with biphasic cartilage-calcium polyphosphate constructs in a sheep model. *Biomaterials*, 27(22), pp. 4120–31. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16564568>. [Accessed July 29, 2010].
- Karageorgiou, V. & Kaplan, D., 2005. Porosity of 3D biomaterial scaffolds and osteogenesis. *Biomaterials*, 26(27), pp. 5474–91. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15860204>. [Accessed August 14, 2013].
- Kim, J. et al., 2014. In vivo performance of combinations of autograft, demineralized bone matrix, and tricalcium phosphate in a rabbit femoral defect model. *Biomedical materials (Bristol, England)*, 9(3), p.035010. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24784998>. [Accessed June 15, 2014].
- Kim, S.-S. et al., 2006. Poly(lactide-co-glycolide)/hydroxyapatite composite scaffolds for bone tissue engineering. *Biomaterials*, 27(8), pp. 1399–409. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16169074>.
- Kobayashi, S., & Murakoshi, T. (2014). Characterization of mechanical properties and bioactivity of hydroxyapatite / β -tricalcium phosphate composites. *Advanced Composite Materials*, 23(2), 163–177.
- Koh, J.L., 2004. The effect of graft height mismatch on contact pressure following osteochondral grafting: a biomechanical study. *American Journal of Sports Medicine*, 32(2), pp. 317–320. Available at: <http://journal.ajsm.org/cgi/doi/10.1177/0363546503261730>. [Accessed August 7, 2013].
- Krishna, B.V., Bose, S. & Bandyopadhyay, A., 2007. Low stiffness porous Ti structures for load-bearing implants. *Acta biomaterialia*, 3(6), pp. 997–1006. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17532277>. [Accessed May 26, 2014].
- Kujala, S. et al., 2003. Effect of porosity on the osteointegration and bone ingrowth of a weight-bearing nickel-titanium bone graft substitute. *Biomaterials*, 24(25), pp. 4691–4697. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0142961203003594>. [Accessed July 29, 2010].
- Lee, J.W. et al., 2009. Development of nano- and microscale composite 3D scaffolds using PPF/DEF-HA and micro-stereolithography. *Microelectronic Engineering*, 86(4–6), pp. 1465–1467. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0167931708006473>. [Accessed June 18, 2014].
- Lee, J.W. et al., 2008. Fabrication and characteristic analysis of a poly(propylene fumarate) scaffold using micro-stereolithography technology. *Journal of Biomedical Materials Research. Part B, Applied Biomaterials*, 87(1), pp. 1–9. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/18335437>. [Accessed July 29, 2010].
- Leong, K., Cheah, C.M. & Chua, C.K., 2003. Solid freeform fabrication of three-dimensional scaffolds for engineering replacement tissues and organs. *Biomaterials*, 24(13), pp. 2363–78. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0142961203000309>.
- Li, J.P. et al., 2005. Porous Ti6Al4V scaffolds directly fabricated by 3D fibre deposition technique: effect of nozzle diameter. *Journal of materials science. Materials in medicine*, 16(12), pp. 1159–63. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16362216>.
- Li, P.J. et al., 2007. Bone ingrowth in porous titanium implants produced by 3D fiber deposition. *Biomaterials*, 28(18), pp. 2810–2820.

- Li, X. et al., 2007. Fabrication of bioceramic scaffolds with pre-designed internal architecture by gel casting and indirect stereolithography techniques. *Journal of Porous Materials*, 15(6), pp. 667–671. Available at: <http://link.springer.com/10.1007/s10934-007-9148-9>. [Accessed June 18, 2014].
- Lin, C.Y., Kikuchi, N. & Hollister, S.J., 2004. A novel method for biomaterial scaffold internal architecture design to match bone elastic properties with desired porosity. *Journal of biomechanics*, 37(5), pp. 623–36. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15046991>. [Accessed April 28, 2014].
- Lin, J.G. et al., 2009. Degradation of the strength of porous titanium after alkali and heat treatment. *Journal of Alloys and Compounds*, 485(1–2), pp. 316–319. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0925838809009724>. [Accessed May 30, 2014].
- Liska, R. et al., 2007. Photopolymers for rapid prototyping. *Journal of Coatings Technology and Research*, 4(4), pp. 505–510. Available at: <http://www.springerlink.com/index/10.1007/s11998-007-9059-3>. [Accessed July 29, 2010].
- Liu, D., et al. (2013a). Mechanical properties' improvement of a tricalcium phosphate scaffold with poly- L -lactic acid in selective laser sintering. *Biofabrication*, 5, 1–10.
- Liu, H. et al., 2012. Simultaneous Reduction and Surface Functionalization of Graphene Oxide for Hydroxyapatite Mineralization. *The Journal of Physical Chemistry C*, 116(5), pp. 3334–3341. Available at: <http://pubs.acs.org/doi/abs/10.1021/jp2102226>.
- Liu, H. & Webster, T.J., 2011. Enhanced biological and mechanical properties of well-dispersed nanophase ceramics in polymer composites: From 2D to 3D printed structures. *Materials Science and Engineering: C*, 31(2), pp. 77–89. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0928493110001761>. [Accessed May 27, 2014].
- Liu, J. et al., 2013. Fabrication and Characterization of Porous 45S5 Glass Scaffolds via Direct Selective Laser Sintering. *Materials and Manufacturing Processes*, 28(6), pp. 610–615. Available at: <http://www.tandfonline.com/doi/abs/10.1080/10426914.2012.736656>. [Accessed June 17, 2014].
- Liu, L. et al., 2009. A Novel Osteochondral Scaffold Fabricated via Multi-nozzle Low-temperature Deposition Manufacturing. *Journal of Bioactive and Compatible Polymers*, 24(1 Suppl), pp. 18–30. Available at: <http://jbc.sagepub.com/cgi/doi/10.1177/0883911509102347>. [Accessed July 6, 2010].
- Long, M., & Rack, H. J. (1998). Titanium alloys in total joint replacement — a materials science perspective. *Biomaterials*, 19, 1621–1639.
- Ma, H. et al., 2012. Preparation and cytocompatibility of polylactic acid/hydroxyapatite/graphene oxide nanocomposite fibrous membrane. *Chinese Science Bulletin*, 57(23), pp. 3051–3058. Available at: <http://link.springer.com/10.1007/s11434-012-5336-3>. [Accessed September 5, 2013].
- Marques, P. a. a. P. et al., 2012. Graphene Oxide and Hydroxyapatite as Fillers of Polylactic Acid Nanocomposites: Preparation and Characterization. *Journal of Nanoscience and Nanotechnology*, 12(8), pp. 6686–6692. Available at: <http://openurl.ingenta.com/content/xref?genre=article&issn=1533-4880&volume=12&issue=8&page=6686>. [Accessed September 5, 2013].
- Matassi, F. et al., 2013. Porous metal for orthopedics implants. Clinical cases in mineral and bone metabolism: the official journal of the Italian Society of Osteoporosis, Mineral Metabolism, and Skeletal Diseases, 10(2), pp. 111–115. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3796997&tool=pmcentrez&rendertype=abstract>.
- Maté-Sánchez de Val, J.E. et al., 2014. Comparison of three hydroxyapatite/ β -tricalcium phosphate/collagen ceramic scaffolds: an in vivo study. *Journal of biomedical materials research. Part A*, 102(4), pp. 1037–46. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23649980>. [Accessed May 27, 2014].
- Matsuno, T. et al., 2008. Preparation of injectable 3 D-formed beta-tricalcium phosphate bead/alginate composite for bone tissue engineering. *Dental materials journal*, 27(6), pp. 827–34. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19241692>.

- Mehrali, M. et al., 2013. Dental implants from functionally graded materials. *Journal of biomedical materials research. Part A*, 101(10), pp. 3046–57. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23754641>. [Accessed November 4, 2013].
- Michiardi, a et al., 2006. New oxidation treatment of NiTi shape memory alloys to obtain Ni-free surfaces and to improve biocompatibility. *Journal of biomedical materials research. Part B, Applied biomaterials*, 77(2), pp. 249–56. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16245290>. [Accessed May 23, 2014].
- Moseke, C. & Gbureck, U., 2010. Tetracalcium phosphate: Synthesis, properties and biomedical applications. *Acta biomaterialia*, 6(10), pp. 3815–23. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20438869>. [Accessed June 15, 2014].
- Mullen, L. et al., 2009. Selective Laser Melting: a regular unit cell approach for the manufacture of porous, titanium, bone in-growth constructs, suitable for orthopedic applications. *Journal of biomedical materials research. Part B, Applied biomaterials*, 89(2), pp. 325–34. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/18837456>. [Accessed June 20, 2014].
- Murr, L.E. et al., 2012. Metal Fabrication by Additive Manufacturing Using Laser and Electron Beam Melting Technologies. *Journal of Materials Science & Technology*, 28(1), pp. 1–14. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S1005030212600164>. [Accessed June 2, 2014].
- Murr, L.E. et al., 2009. Microstructures and mechanical properties of electron beam-rapid manufactured Ti–6Al–4V biomedical prototypes compared to wrought Ti–6Al–4V. *Materials Characterization*, 60(2), pp. 96–105. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S1044580308002076>. [Accessed June 7, 2014].
- Murugan, R. & Ramakrishna, S., 2005. Development of nanocomposites for bone grafting. *Composites Science and Technology*, 65(15–16), pp. 2385–2406. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S026635380500285X>. [Accessed May 30, 2014].
- Navarro, M. et al., 2008. Biomaterials in orthopaedics. *Journal of the Royal Society, Interface / the Royal Society*, 5(27), pp. 1137–58. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2706047&tool=pmcentrez&rendertype=abstract>. [Accessed May 23, 2014].
- Van Noort, R., 2012. The future of dental devices is digital. *Dental materials: official publication of the Academy of Dental Materials*, 28(1), pp. 3–12. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22119539>. [Accessed June 12, 2014].
- Oh, I.-H. et al., 2003. Mechanical properties of porous titanium compacts prepared by powder sintering. *Scripta Materialia*, 49(12), pp. 1197–1202. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S1359646203005244>. [Accessed June 14, 2014].
- Okazaki, Y., 2001. A New Ti–15Zr–4Nb–4Ta alloy for medical applications. *Current Opinion in Solid State and Materials Science*, 5(1), pp. 45–53. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S1359028600000255>.
- Pereira, R. do V. et al., 2014. Scaffolds of PDLLA / Bioglass 58S Produced via Selective Laser Sintering. *Materials Research*, ahead.(ahead).
- Pilliar, R.M. et al., 2007. Osteochondral defect repair using a novel tissue engineering approach: sheep model study. *Technology and Health Care: Official Journal of the European Society for Engineering and Medicine*, 15(1), pp. 47–56. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17264412>.
- Pilliar, R.M. et al., 2013. Porous calcium polyphosphate as load-bearing bone substitutes: in vivo study. *Journal of biomedical materials research. Part B, Applied biomaterials*, 101(1), pp. 1–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23143776>. [Accessed February 13, 2014].
- Pilliar, R.M. et al., 2001. Porous calcium polyphosphate scaffolds for bone substitute applications - in vitro characterization. *Biomaterials*, 22(9), pp. 963–72. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11311015>.
- Pilliar, R.M., Hong, J. & Santerre, P.J., 2009. Method of manufacture of porous inorganic structures, Patent Number 7494614.

- Porter, J.R., Ruckh, T.T. & Popat, K.C., 2009. Bone tissue engineering: a review in bone biomimetics and drug delivery strategies. *Biotechnology progress*, 25(6), pp. 1539–60. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19824042>. [Accessed March 1, 2012].
- Pupo, Y. et al., 2013. Scanning Space Analysis in Selective Laser Melting for CoCrMo Powder. *Procedia Engineering*, 63, pp. 370–378. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S1877705813014410>. [Accessed June 20, 2014].
- Qiu, K. et al., 2006. Fabrication and characterization of porous calcium polyphosphate scaffolds. *Journal of Materials Science*, 41(8), pp. 2429–2434. Available at: <http://link.springer.com/10.1007/s10853-006-5182-2>. [Accessed May 26, 2014].
- Rahaman, M.N. et al., 2011. Bioactive glass in tissue engineering. *Acta biomaterialia*, 7(6), pp. 2355–73. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3085647&tool=pmcentrez&rendertype=abstract>. [Accessed May 27, 2014].
- Reclaru, L. et al., 2005. Electrochemical corrosion and metal ion release from Co-Cr-Mo prosthesis with titanium plasma spray coating. *Biomaterials*, 26(23), pp. 4747–56. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15763254>. [Accessed May 31, 2014].
- Rodríguez-Lorenzo, L., 2009. Synthesis and Biocompatibility of Hydroxyapatite in a Graphite Oxide Matrix. *Key Engineering ...*. Available at: <http://www.scientific.net/KEM.396-398.477>. [Accessed September 3, 2013].
- Rogel, M.R., Qiu, H. & Ameer, G. a., 2008. The role of nanocomposites in bone regeneration. *Journal of Materials Chemistry*, 18(36), p. 4233. Available at: <http://xlink.rsc.org/?DOI=b804692a>. [Accessed June 12, 2014].
- Ryan, G., Pandit, A. & Apatsidis, D.P., 2006. Fabrication methods of porous metals for use in orthopaedic applications. *Biomaterials*, 27(13), pp. 2651–70. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16423390>. [Accessed May 27, 2014].
- Ryan, G.E., Pandit, A.S. & Apatsidis, D.P., 2008. Porous titanium scaffolds fabricated using a rapid prototyping and powder metallurgy technique. *Biomaterials*, 29(27), pp. 3625–35. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/18556060>. [Accessed May 27, 2014].
- Sánchez-Salcedo, S., Nieto, A. & Vallet-Regí, M., 2008. Hydroxyapatite/ β -tricalcium phosphate/agarose macroporous scaffolds for bone tissue engineering. *Chemical Engineering Journal*, 137(1), pp. 62–71. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S1385894707006183>. [Accessed July 29, 2010].
- Sanz-Herrera, J. a, García-Aznar, J.M. & Doblaré, M., 2009. A mathematical approach to bone tissue engineering. *Philosophical transactions. Series A, Mathematical, physical, and engineering sciences*, 367(1895), pp. 2055–78. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19380325>. [Accessed May 18, 2014].
- Shanjani, Y. et al., 2011. Mechanical characteristics of solid-freeform-fabricated porous calcium polyphosphate structures with oriented stacked layers. *Acta Biomaterialia*, 7(4), pp. 1788–96. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21185409>. [Accessed February 11, 2013].
- Shanjani, Y. et al., 2013. Solid freeform fabrication of porous calcium polyphosphate structures for bone substitute applications: in vivo studies. *Journal of biomedical materials research. Part B, Applied biomaterials*, 101(6), pp. 972–80. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23529933>. [Accessed March 12, 2014].
- Shanjani, Y., 2011. Solid freeform fabrication of porous calcium polyphosphate structures for use in orthopaedics.
- Shishkovsky, I.V. et al., 2008. Porous biocompatible implants and tissue scaffolds synthesized by selective laser sintering from Ti and NiTi. *Journal of Materials Chemistry*, 18(12), p.1309. Available at: <http://xlink.rsc.org/?DOI=b715313a>. [Accessed June 20, 2014].
- Shuai, C., Feng, P., Zhang, L., et al., 2013. Correlation between properties and microstructure of laser sintered porous β -tricalcium phosphate bone scaffolds. *Science and Technology of Advanced Materials*, 14(5), p.055002. Available at: <http://stacks.iop.org/1468-6996/14/i=5/a=055002?key=crossref.7011781bc8806989cf2f7e8be93edb63>. [Accessed June 10, 2014].

- Shuai, C., Feng, P., Nie, Y., et al., 2013. Nano-Hydroxyapatite Improves the Properties of (-tricalcium Phosphate Bone Scaffolds. *International Journal of Applied Ceramic Technology*, 10(6), pp. 1003–1013. Available at: <http://doi.wiley.com/10.1111/j.1744-7402.2012.02840.x>. [Accessed May 27, 2014].
- Skog, S. a, Goering, P.L. & Narayan, R.J., 2014. Stereolithography in tissue engineering. *Journal of materials science. Materials in medicine*, 25(3), pp. 845–56. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24306145>. [Accessed May 29, 2014].
- Staiger, M.P. et al., 2006. Magnesium and its alloys as orthopedic biomaterials: a review. *Biomaterials*, 27(9), pp. 1728–34. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16246414>. [Accessed May 24, 2014].
- Stevens, B. et al., 2008. A review of materials, fabrication methods, and strategies used to enhance bone regeneration in engineered bone tissues. *Journal of biomedical materials research. Part B, Applied biomaterials*, 85(2), pp. 573–82. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17937408>. [Accessed July 29, 2010].
- Szpaliski, C., et al. (2012). Bone tissue engineering: current strategies and techniques—Part I: Scaffolds. *Tissue Engineering Part B-Reviews*, 18(4), 246–257.
- Vasiliadis, H.S., Wasiak, J. & Salanti, G., 2010. Autologous chondrocyte implantation for the treatment of cartilage lesions of the knee: a systematic review of randomized studies. *Knee Surgery, Sports Traumatology, Arthroscopy*, 18(12), pp. 1645–1655. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20127071>. [Accessed July 29, 2010].
- Vlasea, M. et al., 2013. A combined additive manufacturing and micro-syringe deposition technique for realization of bio-ceramic structures with micro-scale channels. *The International Journal of Advanced Manufacturing Technology*, 68(9–12), pp. 2261–2269. Available at: <http://link.springer.com/10.1007/s00170-013-4839-7>. [Accessed November 3, 2013].
- Vlasea, M., & Toyserkani, E. (2013). Experimental characterization and numerical modeling of a micro-syringe deposition system for dispensing sacrificial photopolymers on particulate ceramic substrates. *Journal of Materials Processing Technology*, 213(11), 1970–1977.
- Vorndran, E. et al., 2008. 3D Powder Printing of β -Tricalcium Phosphate Ceramics Using Different Strategies. *Advanced Engineering Materials*, 10(12), pp.B67–B71. Available at: <http://doi.wiley.com/10.1002/adem.200800179>. [Accessed August 20, 2013].
- Wataha, J. C., et al. (2001). Relating Nickel-Induced Tissue Inflammation to Nickel Release in vivo. *Journal of Biomedical Materials Research*, 58(5), 537–544.
- Weber, J. N., & White, E. W. (1972). Carbon-metal graded composites for permanent osseous attachment of non-porous metals. *Materials Research Bulletin*, 7(9), 1005–1016.
- Wei, G. & Ma, P.X., 2004. Structure and properties of nano-hydroxyapatite/polymer composite scaffolds for bone tissue engineering. *Biomaterials*, 25(19), pp. 4749–57. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15120521>.
- Wen, C.E. et al., 2002. Novel titanium foam for bone tissue engineering. *JOURNAL OF MATERIALS RESEARCH*, 17 10 2633-2639.
- Will, J. et al., 2008. Porous ceramic bone scaffolds for vascularized bone tissue regeneration. *Journal of Materials Science: Materials in Medicine*, 19(8), pp. 2781–90. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/18305907>. [Accessed August 22, 2013].
- Woesz, A., 2008a. Scaffolds with controlled architecture for possible use in bone tissue engineering.
- Woesz, A., 2008b. Scaffolds with controlled architecture for possible use in bone tissue engineering.
- Woesz, A., et al. (2005). Towards bone replacement materials from calcium phosphates via rapid prototyping and ceramic gelcasting. *Materials Science and Engineering C*, 25, 181–186.
- Wong, K. V & Hernandez, A., 2012a. A Review of Additive Manufacturing.,
- Wong, K. V & Hernandez, A., 2012b. A Review of Additive Manufacturing. *International Scholarly Research Network Mechanical Engineering*, 2012, pp. 1–10.
- Xiong, Z. et al., 2002. Fabrication of porous scaffolds for bone tissue engineering via low-temperature deposition. *Scripta Materialia*, 46(11), pp. 771–776. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S1359646202000714>.

- Yang, S. et al., 2001. The design of scaffolds for use in tissue engineering. Part I. Traditional factors. *Tissue Engineering*, 7(6), pp. 679–89. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11749726>.
- Yang, S., Du, Z., & Chua, C. K. (2002). Review The Design of Scaffolds for Use in Tissue Engineering. *Part II. Rapid Prototyping Techniques. Tissue Engineering*, 8(1), 1–11.
- Yarlagadda, P.K.D. V, Chandrasekharan, M. & Shyan, J.Y.M., 2005. Recent advances and current developments in tissue scaffolding. *Bio-medical materials and engineering*, 15(3), pp. 159–77. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15911997>.
- Zanin, H. et al., 2013. Fast preparation of nano-hydroxyapatite/superhydrophilic reduced graphene oxide composites for bioactive applications. *Journal of Materials Chemistry B*. Available at: <http://xlink.rsc.org/?DOI=c3tb20550a>. [Accessed August 29, 2013].
- Zhang, K. et al., 2004. Processing and properties of porous poly(l-lactide)/bioactive glass composites. *Biomaterials*, 25(13), pp. 2489–2500. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0142961203007695>. [Accessed May 28, 2014].
- Zhang, L. et al., 2013. A tough graphene nanosheet/hydroxyapatite composite with improved in vitro biocompatibility. *Carbon*, 61, pp. 105–115. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0008622313003837>. [Accessed June 2, 2014].
- Zhang, Y., et al. (2003). Calcium Phosphate – Chitosan Composite Scaffolds for Bone. *Tissue Engineering*, 9(2).
- Zhou, W.Y. et al., 2008. Selective laser sintering of porous tissue engineering scaffolds from poly(L: -lactide)/carbonated hydroxyapatite nanocomposite microspheres. *Journal of materials science. Materials in medicine*, 19(7), pp. 2535–40. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17619975>. [Accessed June 6, 2014].

3D Printing of Cartilage and Subchondral Bone

14

Shawn P. Grogan, Erik W. Dorthé, Joel Kopcow and Darryl D. D'Lima

Shiley Center for Orthopaedic Research and Education at Scripps Clinic, La Jolla, CA, United States

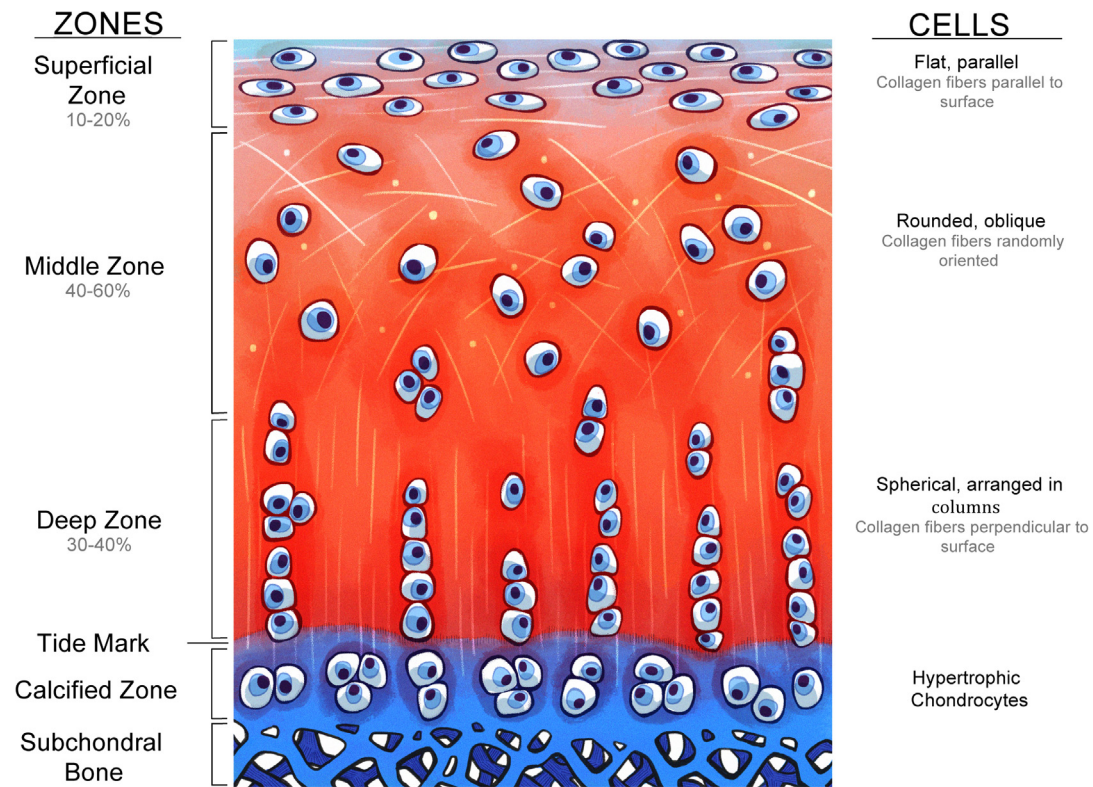
14.1 BACKGROUND

Cartilage and subchondral bone are unique tissues that correspond to their mechanical and load-bearing function. Cartilage is a remarkable living tissue that permits almost frictionless articulation in synovial joints and protects the subchondral bone from mechanical stress. To fully utilize any potential bioprinting approach or technology, an understanding of the structure, organization, and function of these tissues is essential.

14.1.1 FUNCTION AND ORGANIZATION

Articular cartilage is a load-bearing tissue devoid of neural innervation, vasculature, or a lymphatic system and comprises chondrocytes that produce and maintain an extensive extracellular matrix (ECM) network. The major components of the cartilaginous matrix include collagen fibrils and glycosaminoglycans (GAGs), which bind much of the water that makes up 70%–80% of cartilage. Collagen type II is the predominant collagen, but other collagens such as types VI, IX, XI, and XIV are also present in small quantities. Other noteworthy macromolecules that make up the cartilage ECM include link protein, cartilage oligomeric matrix protein (COMP), decorin, tenascin, fibromodulin, and fibronectin (Hunziker, Michel, & Studer, 1997). Articular cartilage is not a simple mixture of ECM components; rather it is exquisitely structured and organized into “zones” of cells and ECM compositions extending from the articular surface transitioning down to the underlying subchondral bone (Fig. 14.1). Zonal variations in the collagen network orientation and depth-dependent variation in the concentration of GAGs contribute to the complex compressive, shear, and tensile properties (Benninghoff, 1925; Schinagel, Gurskis, Chen, & Sah, 1997).

The upper, most specialized zone (upper 10–50 μm or 10% of the full thickness, Fig. 14.1) is the superficial zone (SZ) that experiences both compressive and shear forces (Buckley, Gleghorn, Bonassar, & Cohen, 2008; Wong, Kim, Antonacci, McIlwraith, & Sah, 2010), harbors the highest cell density, and contains cells that are small, flattened, and without an extensive pericellular matrix (Hunziker, Quinn, & Hauselmann, 2002; Siczkowski & Watt, 1990). SZ cells secrete a number of unique proteins including a “lubricant” molecule known as SZ protein or lubricin (Flannery et al., 1999; Jay, Haberstroh, & Cha, 1998; Schumacher, Hughes, Kuettner, Caterson, & Aydelotte, 1999), clusterin (Khan, Salter, Bayliss, Thomson, & Archer, 2001; Malda et al., 2010), and

**FIGURE 14.1**

Articular cartilage and subchondral bone organization, cellular arrangements, morphology, and unique features of zonal extracellular matrix components.

developmental endothelial locus-1 (Del1) (Pfister, Aydelotte, Burkhart, Kuettner, & Schmid, 2001). In the SZ, chondrocytes secrete less collagen type II (Darling, Hu, & Athanasiou, 2004; Hidaka, Cheng, Alexandre, Bhargava, & Torzilli, 2006; Khan et al., 2001) and the ECM contains the least amount of GAGs (Buckwalter & Mankin, 1998). The SZ cells, however, produce higher levels of keratan sulfate and other specialized proteoglycans in comparison with the deep zone (DZ) cells (Aydelotte, Greenhill, & Kuettner, 1988; Aydelotte & Kuettner, 1988; Siczkowski & Watt, 1990; Waldman, Grynpsas, Pilliar, & Kandel, 2003; Zanetti, Ratcliffe, & Watt, 1985). The specialized proteoglycans known as small leucine-rich proteoglycans (Miosge et al., 1994; Poole et al., 1996) are found in higher concentrations in the SZ including decorin, biglycan, asporin, lumican, and fibromodulin that modulate many processes including metabolic processes, growth factor signaling, and maintaining tissue integrity (Kizawa et al., 2005; Pelletier et al., 2000; Poole et al., 1996; Roughley, 2006). A fibulin-like ECM protein (EFEMP1) was found predominantly expressed in the SZ (Grogan, Duffy, et al., 2013) and was postulated to maintain the immature status of chondroprogenitor cells (Wakabayashi et al., 2010), which is consistent with the presence of progenitor cell

populations residing in this region (Alsalameh, Amin, Gemba, & Lotz, 2004; Dowthwaite et al., 2004; Grogan, Miyaki, Asahara, D'Lima, & Lotz, 2009) and appears critical for replenishing cells and maintaining tissue homeostasis.

The middle zone (MZ) (40%–60% of cartilage thickness; Fig. 14.1) contains chondrocytes with more pericellular matrix and collagen fibrils that are more randomly dispersed. In comparison with the SZ, the MZ contains higher levels of aggrecan (Bayliss, Venn, Maroudas, & Ali, 1983) and is specifically high in hyaluronic acid (HA), dermatan sulfate, and collagen type II (Asari et al., 1994; Buschmann, Maurer, Berger, Perumbuli, & Hunziker, 2000; Franzen, Inerot, Hejderup, & Heinegard, 1981; Maroudas, Muir, & Wingham, 1969; Ratcliffe, Fryer, & Hardingham, 1984; Schinagl et al., 1997; Wong, Wuethrich, Egli, & Hunziker, 1996). The main role of aggrecan is to maintain tissue hydration, which is important for endowing cartilage its mechanical properties (Dudhia, 2005). Cartilage intermediate layer protein is uniquely located in the MZ (Lorenzo, Bayliss, & Heinegard, 1998). The ECM protein COMP is also mainly seen in MZ and DZ (DiCesare, Morgelin, Carlson, Pasumarti, & Paulsson, 1995; Murray, Smith, Henson, & Goodship, 2001).

The deepest 30%–40% of cartilage thickness is classified as the radial or DZ (Fig. 14.1) that contains the lowest cell density and collagen content compared with the SZ and MZ (Venn, 1978). On the other hand, the proteoglycan content, including aggrecan, keratan sulfate, and chondroitin sulfate, is much higher than the other zones (Asari et al., 1994; Jacoby & Jayson, 1975; Mitrovic & Darmon, 1994; Muir, Bullough, & Maroudas, 1970). Although the total collagen content in the DZ is lower than that in the other zones, the collagen fibrils are larger in diameter. The cells are commonly arranged into stacks or columns and, along with the collagen fibrils, are oriented perpendicular to the articular surface (Siczkowski & Watt, 1990) (Fig. 14.1). Osteopontin is exclusively expressed in the DZ (Pullig, Weseloh, Ronneberger, Kakonen, & Swoboda, 2000; Schnapper & Meyer, 2004).

Integration between articular cartilage and the much stiffer subchondral bone begins at the base of the DZ where a 5- μ m thick undulating “tidemark” is observed, which marks the transition into a layer of calcified cartilage (20–250- μ m thick) containing perpendicular chondrocyte-derived collagen type II fibers that become structurally cemented in the collagen type I osteoid produced by osteoblasts (Hoemann, Lafantaisie-Favreau, Lascau-Coman, Chen, & Guzman-Morales, 2012; Orth, Cucchiari, Kohn, & Madry, 2013) (Fig. 14.1). The calcified cartilage contains small cells in a chondroid matrix that is characterized by increased mineral density speckled with apatitic salts (Burr, 2004; Mow, Proctor, & Kelly, 2012).

This unique zonal and layered composition of osteochondral tissue makes it an ideal candidate for three-dimensional (3D) bioprinting. The five primary layers (subchondral bone, calcified cartilage, DZ, intermediate zone, and SZ) contain different cell types and matrix components. Bioprinting each of these zones with unique cell and matrix compositions will better emulate and potentially enhance the development of osteochondral tissue. Placement of progenitor cell populations surrounded by specific bioinks composed of regulatory stem cell niches may enhance longer term tissue survival and repair.

14.1.2 INJURY, DISEASE, AND TREATMENT

Cartilage function is often compromised following acute or chronic injury or as part of the aging process and eventually leading to tissue degeneration manifested as osteoarthritis (OA). For patients older than 60 years of age with end-stage arthritis, artificial joint replacement is the recommended treatment

(Grayson & Decker, 2012; Noble et al., 2005; Wylde, Livesey, & Blom, 2012). For younger patients, biological or cell-based procedures are recommended such as microfracture (Goyal, Keyhani, Lee, & Hui, 2013; Goyal et al., 2014; Sherman et al., 2014), autologous chondrocyte implantation (Kon, Filardo, Di, & Marcacci, 2012), or osteochondral grafting (Sherman et al., 2014). Microfracture involves piercing the subchondral bone to induce bleeding and clot formation which matures into fibrocartilaginous tissue (Mithoefer et al., 2005). In autologous chondrocyte implantation, chondrocytes are harvested from a biopsy of normal cartilage and expanded in culture (Brittberg et al., 1994). In a second procedure, these chondrocytes in suspension are placed underneath a periosteal or collagen membrane covering the lesion. Osteochondral grafting involves implanting healthy bone and cartilage grafts to replace the lost tissue (Gortz & Bugbee, 2006; Marcacci et al., 2007). However, these approaches do not result in durable repair in the long term with the major issues related to the lack of regeneration of the zonal structure of hyaline articular cartilage and poor integration with host tissue (Clar et al., 2005; Devitt, Bell, Webster, Feller, & Whitehead, 2017; Rasanen et al., 2007; Zeifang et al., 2010).

Over the past 15 years, much effort has been devoted to the prefabrication of neotissues using various scaffold systems and bioreactors (Tuan, Chen, & Klatt, 2013). These tissue engineering approaches typically involve cell harvesting and expansion, followed by an *in vitro* phase of 3D culture to produce a neotissue graft (Roelofs, Rocke, & De Bari, 2013). The general concept of maturing a cultured 3D graft tissue before implantation is to ensure the development of cartilage matrix with appropriate mechanical properties (Mohanraj, Farran, Mauck, & Dodge, 2013; Pabbruwe, Esfandiari, Kafienah, Tarlton, & Hollander, 2009; Theodoropoulos, De Croos, Park, Pilliar, & Kandel, 2011). However, integration of mature cartilage with the host tissue is limited or not consistently demonstrated due to a lack of vascularity, mismatch between the mechanical properties of the native tissue and implanted graft, cell death, inadequate differentiation of the cells, and the type of biomaterial or scaffold used (Khan, Gilbert, Singhrao, Duance, & Archer, 2008). Efforts toward translating engineered cartilage tissue into human clinical trials have been initiated, such as Denovo ET (Engineered Tissue Graft) and NeoCart (see clinicaltrials.gov). Outcomes of the NeoCart phase-II prospective, randomized clinical trial were similar to those after microfracture and were associated with greater clinical efficacy at 2 years after treatment (Crawford, DeBerardino, & Williams, 2012; Fedorovich et al., 2012). Autologous chondrocytes cultured on porcine collagen membrane (MACI) were recently approved by the FDA for treating cartilage defects with promising clinical results at 5 years (Brittberg, Recker, Ilgenfritz, Saris, & Group, 2018). However, longer term clinical outcomes are pending.

Overall, the current strategies to engineer cartilage have failed to fabricate new repair tissue *in vivo* that replicates native cartilage in terms of mechanical properties, zonal organization, and ECM (Klein et al., 2009; Schuurman, Levett, et al., 2013). The characterization of cartilage biology and ECM in terms of structure and organization (Grogan et al., 2009; Miosge et al., 1994; Poole et al., 1996; Waldman et al., 2003), cartilage zonal phenotype (Grogan, Duffy, et al., 2013) and the availability of promising cells sources [e.g., mesenchymal stem cells (MSC) and ESC] (Bulman, Barron, Coleman, & Barry, 2013; Filardo et al., 2013; Olee et al., 2014) coupled with advances in biomaterials (Spiller, Maher, & Lowman, 2011; Tuan et al., 2013) are collectively advancing the field toward the effective osteochondral repair. 3D bioprinting is an emerging technology with the ability to deliver cells and matrix proteins to form repair tissues with the required cell distributions, zones, and variation in ECM to mimic normal articular cartilage. If successful, 3D bioprinting can overcome the major hurdles preventing successful repair such as poor reproduction of the tissue structure and organization and weak integration with host tissue.

14.2 APPLICATIONS OF 3D PRINTING

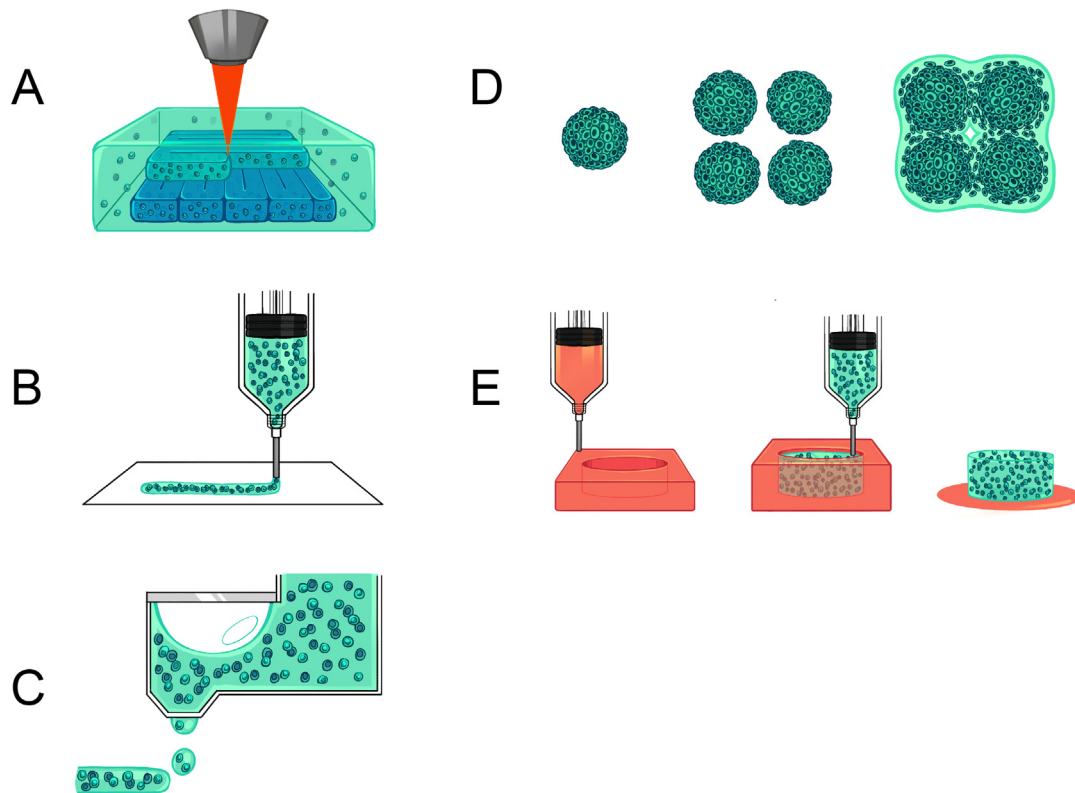
A number of techniques have been developed for 3D bioprinting of tissues in general. These printing systems are described in more detail in Chapter 2, Bioprinting of Biomimetic Tissue Models for Disease Modeling and Drug Screening, and Chapter 3, 3D Bioprinting Techniques. In this chapter, we focus on the 3D bioprinting of cartilage and subchondral bone.

All techniques share a key characteristic: the reliance on some form of printable material often called a “bioink.” This can take many forms; in the simplest case, it is a biocompatible, printable fluid. This bioink is intended as a carrier for cells and provides a scaffold material for tissue engineering. In these instances, the bioink must provide both structure for the 3D-printed construct and a favorable environment for cell viability. Hydrogels form a major component of many bioinks (see Section 14.3.2). Typically, cells are suspended in the bioink (Cui, Breitenkamp, Finn, Lotz, & D’Lima, 2012; Cui, Breitenkamp, Lotz, & D’Lima, 2012). Alternatively cells may be clustered into spheroids or other microstructures to enable printing with small, preassembled tissue-like constructs which can decrease the time necessary for cells to self-organize after printing (Grogan, Dorthé, Glembotski, Gaul, & D’Lima, 2020; Mironov et al., 2009; Norotte, Marga, Niklason, & Forgacs, 2009).

Of the various 3D-printing technologies, the earliest was stereolithography (Billiet, Vandenhaute, Schelfhout, Van Vlierberghe, & Dubruel, 2012). In stereolithography, a laser is rastered over a reservoir of photocrosslinkable polymer, solidifying the material (Fig. 14.2A). After each layer, the printing substrate is lowered or the reservoir depth is increased, and the process is repeated to form each layer in the object. This can be easily adapted to photocrosslinkable biomaterials and has been used for implant and scaffold creation (Billiet et al., 2012; Grogan, Chung, et al., 2013; Soman, Chung, Zhang, & Chen, 2013). Cells suspended in the bioink are encased within the hydrogel matrix at the time of crosslinking and usually survive the relatively brief ultraviolet laser exposure during printing (Lin et al., 2013). Instead of a moving laser, 2D projection techniques, for example with digital micromirrors, can dramatically increase the print speed while preserving resolution and limiting cell exposure to ultraviolet light (Beke et al., 2012; Grogan, Chung, et al., 2013). However, stereolithography requires a relatively large amount of bioink in the reservoir, and the feasibility for multi-ink printing is limited (Melchels et al., 2012).

Extrusion printing is the most common 3D-printing technology, very similar to consumer-grade plastic 3D printers. For bioprinting applications, the cells suspended in a bioink are loaded into a delivery system and extruded to build layers (Fig. 14.2B). The relatively large nozzle sizes and high extrusion pressure permit the extrusion of viscous inks with high cell density. One disadvantage is that large nozzle sizes and bulk extrusion volumes limit the resolution of the print.

Inkjet bioprinting draws on the same technology as inkjet document printing. A low viscosity fluid is ejected from a nozzle toward the printing substrate, typically by a thermal bubble or a piezoelectric effect (Fig. 14.2C). Both thermal and piezoelectric inkjets are capable of printing viable cells (Cui, Boland, D’Lima, & Lotz, 2012; Cui, Breitenkamp, Finn, et al., 2012; Cui, Breitenkamp, Lotz, et al., 2012; Saunders, Gough, & Derby, 2008; Tirella et al., 2011). The print nozzles do not require direct contact with the print substrate or previously printed layers, which can be an advantage in certain medical applications. Incorporating multiple inkjet print cartridges makes it feasible to construct complex tissues containing multiple cell types (Xu et al., 2013). The small size of individual droplets increases the resolution of the printed constructs. However, this higher resolution

**FIGURE 14.2**

Overview of the mechanisms of action for each 3D-printing method. (A) Stereolithography traps cells that are suspended in a liquid photosensitive monomer hydrogel. (B) Extrusion pushes suspended cells from a column through a contacting nozzle. (C) Inkjets use a transient pressure increase to eject a droplet of bioink. (D) Matrix-free methods assemble microtissues in close proximity to encourage tissue fusion and generate neotissue. (E) Indirect methods use sacrificial supports or molds to improve shape retention and resolution.

comes at the cost of increased print duration and limits the application to low viscosity inks. Cell settling and clumping can also clog the microchannels and nozzles in an inkjet system (Chahal, Ahmadi, & Cheung, 2012).

“Freeform Reversible Embedding of Suspended Hydrogels” (FRESH) and melt-electrowriting address some of the challenges in maintaining the printed structure of many bioinks, especially the softer hydrogels. FRESH involves extruding hydrogel inks into a thermoreversible slurry of gelatin microparticles that support the printed structures (Hinton et al., 2015). Melt-electrowriting is an electrohydrodynamic process using a high voltage electrical field to deposit molten polymer (PCL) with diameters less than a tenth of that achieved by extrusion printing (de Ruijter, Ribeiro, Dokter, Castilho, & Malda, 2019; Diloksumpan et al., 2020; Peiffer et al., 2020). Soft hydrogel bioinks can then be coprinted into the PCL scaffold.

14.2.1 3D-PRINTING CARTILAGE AND SUBCHONDRAL BONE

Cartilage 3D printing has come a long way from printing scaffolds that were subsequently seeded with cells. One early example was the TheriForm™ process that bound a mixture of PLGA and PLA polymer powder particles with chloroform to generate a porous scaffold that was subsequently seeded with cells (Sherwood et al., 2002). In another example, poly(ethylene glycol)-terephthalate–poly(butylene terephthalate) (PEGT/PBT) copolymer was extruded to print scaffolds, into which bovine articular chondrocytes were seeded (Schuurman, Harimulyo, et al., 2013). Others have reported printing PEOT/PBT (Moroni et al., 2008); poly(ester urea) (PEU) (Moxon et al., 2020); natural polymers such as silk fibroin (Shi et al., 2017); or combinations of natural and artificial polymers (fibrin, HA, collagen, and PCL) (Visser et al., 2016). In contrast, bioprinting involving simultaneous printing of cells and scaffolds has several advantages. Perhaps the most important advantage is the spatial distribution of cells which can be a challenge to control when seeding pre-fabricated scaffolds (Carletti, Motta, & Migliaresi, 2011; Wendt, Stroebel, Jakob, John, & Martin, 2006). Alternatively, scaffold-free fabrication involves printing just cells, preformed cells clusters, or microtissues.

14.2.1.1 Extrusion Printing

Extrusion-based printers are most frequently used for cartilage 3D printing given the commercial availability, ease of use, and versatility in dispensing hydrogel bioinks and cells. The printed constructs can be stabilized by several methods (Lu et al., 2018) including temperature (Abbadessa et al., 2016; Young, White, & Daniele, 2020), photocrosslinking (Gao et al., 2021; Van Hoorick et al., 2019), chemical (Sakai, Ueda, Gantumur, Taya, & Nakamura, 2018), ionic (Piras & Smith, 2020), and enzymatic actions (Weisel & Litvinov, 2017). Fedorovich et al. used a pneumatic dispensing system (BioScaffolder) to deposit human chondrocytes and bone marrow-derived MSC in alginate and reported cartilage formation, albeit limited bone formation, after implantation in nude mice (Fedorovich et al., 2012). Since then, there have been numerous reports on extrusion-based bioprinting of cartilage or osteochondral tissues. Rat chondrocytes in sodium alginate were printed in grid-like patterns and crosslinked with CaCl_2 in a 3D-Bioplotter system (Regenovo, Zhejiang, China) (Yang et al., 2018). Combining collagen or agarose with the alginate improved the mechanical strength, while the alginate + collagen constructs resulted in better cell attachment, proliferation, and cartilage tissue formation. Similar beneficial effects were also reported in a study using human adipose-derived stem cells (ADSC) and a photocrosslinkable alginate bioconjugated with gelatin and chondroitin sulfate to mimic the cartilage ECM (Olate-Moya et al., 2020). Combining alginate with gelatin and fibrinogen also supported chondrogenic differentiation of MSC when stimulated with TGF β 1 and BMP2 (Henrionnet et al., 2020).

Soft hydrogels such as methacrylated gelatin (GelMA) or alginate require thickeners to improve printability. Gellan gum added to GelMA enhanced viscosity and speed of gelation (Melchels, Dhert, Huttmacher, & Malda, 2014). A formulation of 0.5% gellan gum in 10% GelMA provided the optimal combination of printability, fidelity, and mechanical properties to support cartilage tissue formation by equine chondrocytes (Mouser et al., 2016). Similarly, adding nanofibrillated cellulose to alginate improved printability and postprint construct stability in cartilage constructs (Markstedt et al., 2015; Muller, Ozturk, Arlov, Gatenholm, & Zenobi-Wong, 2017). Human MSC in various combinations of GelMA, chondroitin sulfate amino ethyl methacrylate (CS-AEMA), and

methacrylated hyaluronic acid (HAMA) were mixed with alginate. A coaxial-dispensing system delivered the bioink in the inner needle and calcium chloride in the outer needle to stabilize the printed fibers, which were subsequently photocrosslinked after printing (Costantini et al., 2016). The combination of GelMA and CS-AMEA induced greater chondrogenic differentiation while adding HAMA induced chondrocyte hypertrophy.

Another multicomponent hydrogel (GelXA Fibrin, CELLINK, Boston MA), which is a blend of fibrinogen, GelMA, xanthan gum, sodium alginate, and the photoinitiator LAP, combines photocrosslinking (UV light), ionic (CaCl_2) and enzymatic (thrombin) crosslinking to enhance the mechanical properties of the gel. We showed results of bioprinting ESC–MSC in the INKredible printer (CELLINK) into chondral lesions created in human osteoarthritic tissue (Fig. 14.3).

Silk fibroin has promise as a printable material with attractive mechanical properties and biodegradability (Brenckle et al., 2013; Zhang et al., 2018). Combining fibroin with gelatin, the affinity peptide E7 (E7), or platelet-rich plasma (PRP) can overcome some of the issues encountered with attachment and proliferation of bone marrow-derived mesenchymal stem cells (BMMSC) (Shi et al., 2017; Wang et al., 2015). This combination, when implanted into rabbit osteochondral defects, resulted in significantly better cartilage regeneration compared with microfracture. More recently

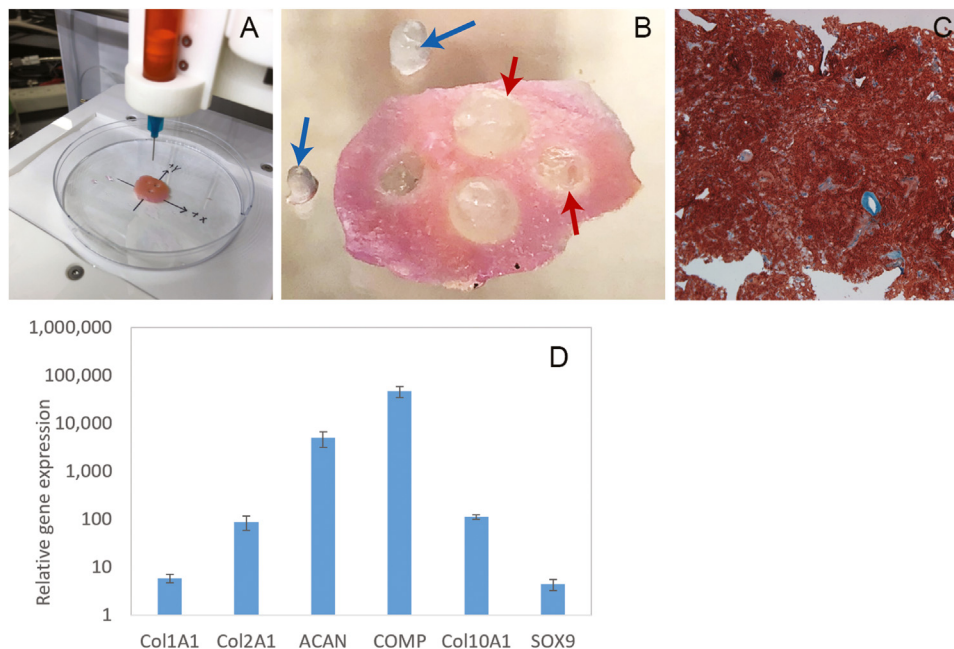


FIGURE 14.3

In situ extrusion printing. ES-MSC (20×10^6 cells/mL) suspended in a fibrin-based gel were printed into *ex vivo* human OA cartilage explants. (A) *Ex vivo* tissue with multiple defects on printer stage. (B) Defects filled with *in situ* printed bioink (red arrows) along with free-standing printed constructs (blue arrows). (C) Safranin O staining after 3 weeks in culture showing high GAG content (magnification $10 \times$). (D) Chondrogenic gene expression increased significantly (relative to ES-MSC in monolayer culture).

rabbit chondrocytes seeded on scaffolds of fibroin, polyethylene glycol (PEG), and PRP were able to regenerate cartilage; with PRP generating a dose-dependent increase in chondrogenic gene expression (COL2A1 and ACAN) and neotissue formation (collagen type II and GAGs) (Li et al., 2020).

Another method of enhancing the stability of printed constructs is to employ a dual or a coprinting approach. Stiffer polymers, such as PCL and PLA, can serve as structural components for softer hydrogel bioinks, such as agarose, alginate, GelMA, or HAMA. PCL fibers printed in a grid-pattern provided spaces for deposition of BMMSC encapsulated in a hydrogel (gelatin, fibrin, HA, and glycerol) (Sun, You, Jiang, Zhai, & Dai, 2019). These constructs regenerated cartilage tissue when implanted in full-thickness cartilage defects in rabbits. MSC printed in agarose and alginate supported chondrogenic differentiation and codeposition of PCL filaments in the hydrogel and increased the bulk compressive moduli comparable to articular cartilage (Daly, Critchley, Rencsok, & Kelly, 2016). Similarly, PLA coprinted with HA-based bioink supported neotissue formation by chondrocytes with enhanced mechanical properties (Antich et al., 2020).

The zonally organized nature of articular cartilage lends itself to the layer-by-layer fabrication process by which most 3D-printing platforms operate. Upper layers of articular cartilage progenitor cells (ACPC) were deposited on lower layers of BMMSC or chondrocytes in GelMA (Levato et al., 2017). After 8 weeks in culture, cartilage-like tissues were formed with the upper zone containing more SZ protein, and the lower zone containing greater GAGs and collagen type II. Gellan gum and HAMA were added to GelMA to improve print fidelity (Mouser et al., 2020). A bioprinted two-zone construct with the SZ containing ACPC and the lower zone containing MSC produced neo-cartilage-like tissues over 28 days of culture. No specific zonal phenotype was noted between the two regions, although the MSC produced slightly more GAGs compared to the ACPCs.

Printing of both cartilage and bone (osteocondral printing) is actively being pursued to treat clinical articular defects which often extend into subchondral bone. Almost all approaches involve a combination of multiple materials and cell types for the cartilage and bone phase. Acellular scaffolds have been printed that were subsequently seeded with MSC (Holmes, Zhu, Li, Lee, & Zhang, 2015) or chondrocytes (Moxon et al., 2020) or were able to recruit endogenous cells after implantation (Gao et al., 2021). Using bioprinting, a multiheaded extrusion printer was used to print a multi-layered 3D construct with PCL (Shim et al., 2016). MSCs were encapsulated in collagen to form the lower subchondral bone layer and in a modified HA hydrogel to form the upper chondral layer. The printed constructs were implanted in osteochondral defects in rabbit knees showing evidence of bone and cartilage healing. PCL, PLA, and PLGA fiber networks have been explored to reinforce biphasic alginate or agarose hydrogels seeded with several cell types including chondrocytes, MSC from the infrapatellar fat pad, and BMMSC (Critchley et al., 2020). Various combinations of materials and cells were implanted subcutaneously in nude mice and evaluated for the development of osteochondral tissues. The most promising biphasic construct consisted of an osseous layer of BMMSC mixed in alginate and seeded into a PCL fiber network and a chondral layer of cocultured chondrocytes and fat pad MSC. Implantation of these constructs into osteochondral defects in goat knees resulted in promising regeneration of cartilage and subchondral bone healing after 6 months.

Calcium salts, such as hydroxyapatite, are attractive for printing the bone phase of osteochondral constructs. A methacrylated gellan gum-based bioink, mixed with either HAMA or hydroxyapatite particles, has been proposed for cartilage and bone regeneration, respectively (Muller et al., 2020). In a multiscale approach, melt-electrowriting of spatially organized PCL microfibers and extrusion printing of a bone-biomimetic ceramic ink (α -tricalcium phosphate and

nanohydroxyapatite) were used to fabricate a subchondral bone scaffold into which MSCs were seeded (Diloksumpan et al., 2020). ACPs in GelMA were layered on top to develop the chondral tissue. Both scaffolds supported osteogenic and chondrogenic matrix formation in culture.

Cryogenic 3D printing involves rapidly cooling the ink during printing to below freezing temperature. Biphasic osteochondral scaffolds were cryo-printed with β -tricalcium phosphate/poly(lactic-co-glycolic acid) in the bone phase and poly(D,L-lactic acid-co-trimethylene carbonate) in the chondral phase; the latter then loaded with collagen I hydrogel containing TGF β 1 (Wang et al., 2020). Rat BMMSC cultivated in each layer showed evidence of chondrogenic and osteogenic differentiation.

14.2.1.2 Inkjet Printing

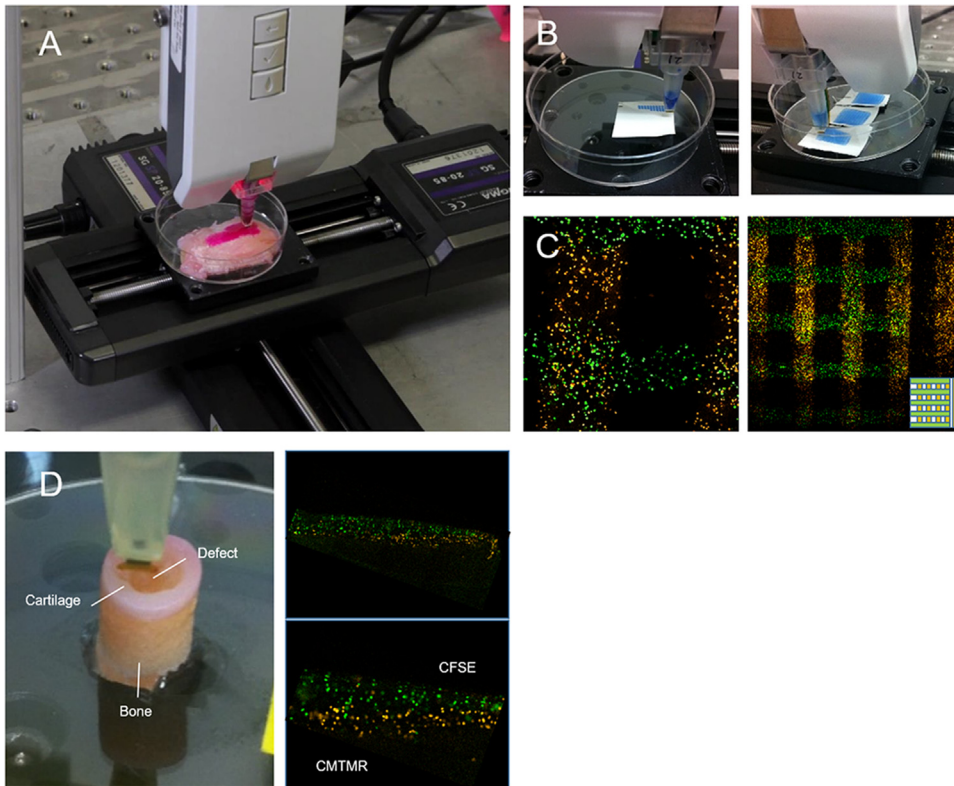
Inkjet or droplet-based cartilage bioprinting was reported in the early 2000s (Wilson & Boland, 2003). We have shown that thermal inkjet deposition of polyethylene glycol dimethacrylate (PEGDA) containing human chondrocytes showed promise for *in situ* cartilage repair in osteochondral explants (Cui, Breitenkamp, Finn, et al., 2012; Cui, Breitenkamp, Lotz, et al. 2012). The Thermal Ink Jet Pico-Fluidic System (TIPS, Hewlett-Packard Inc, Corvallis, OR) can dispense precise liquid volumes in the nano- and picoliter range (Fig. 14.4) (Stasiak, 2017). We mounted the TIPS system on an XYZ stage and printed cells in PEGDA and alginate. By optimizing the voltage and pulse duration we were able to print cells in precise patterns on electrospun biopaper. For proof-of-concept of multimaterial printing, we printed patterns containing cells stained with different colors in layers within a defect created osteochondral explants.

Thermal inkjets have high accuracy and resolution but can be limited to low viscosity inks with low cell density (e.g., less than 10×10^6 cells/mL of 0.5% alginate). A 3D printer with a static piezoelectric-actuated droplet generator dispensed ovine MSC at a cell density of 10×10^6 cells/mL which generated cartilage-like tissues after 35 days of culture (Graham et al., 2017). We used a pneumatically driven diaphragm within a specially formed nozzle chamber to generate the pressure to eject an inkjet droplet. By selecting the material and thickness of the diaphragm, and the size of the print nozzle, we could print with cell densities of 20×10^6 /mL in bioink containing up to 10% collagen solutions.

14.2.1.3 Scaffold-free Printing

Using cellular microspheroids or microtissues is an alternative approach to bioprinting cells suspended in bioinks or into scaffolds (Fig. 14.2D). Major advantages include elimination of issues related with scaffolds and bioinks such as biocompatibility and biomaterial degradation; and feasibility of achieving a very high cell density.

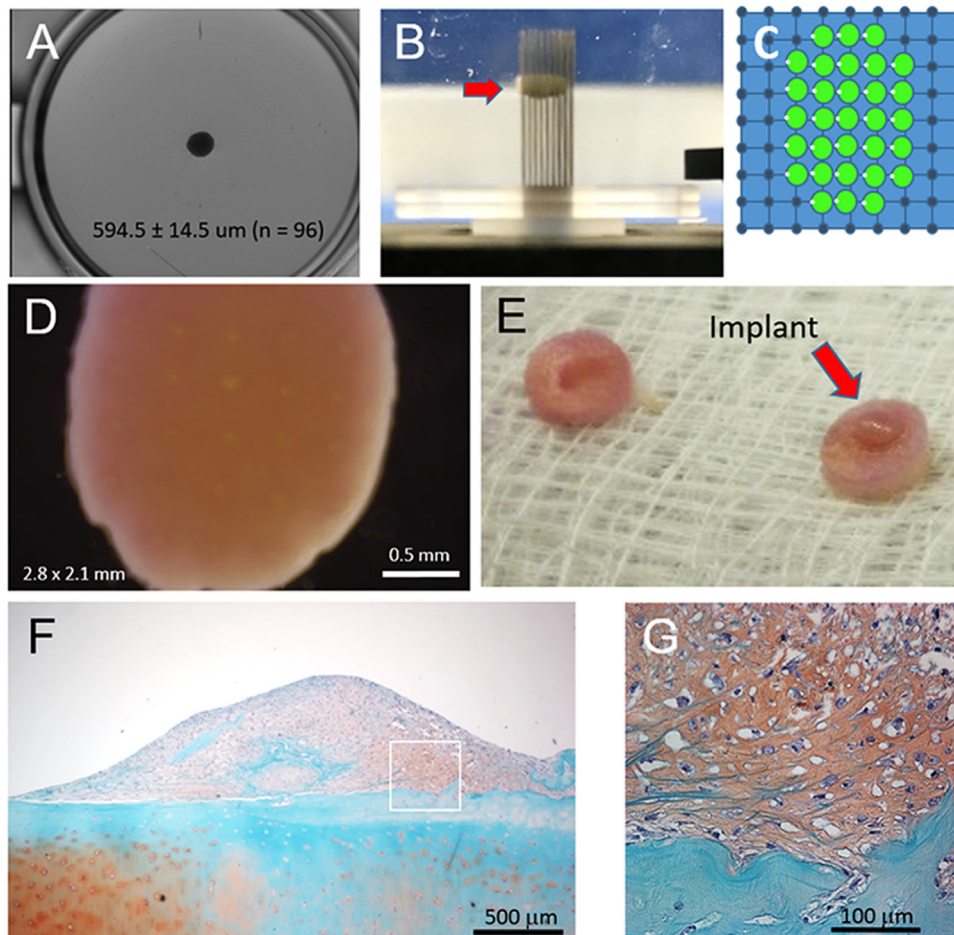
The so-called “Kenzan” method involves assembling cellular microspheroids on an array of microneedles (Moldovan, Hibino, & Nakayama, 2017). We used the Kenzan-based Regenova system (Cyfuse Biomedical, Tokyo, Japan) to print constructs from microspheroids of human ESC–MSC and human infrapatellar fat pad stem cells. Fig. 14.5 provides an overview of creating cartilaginous tissue from microspheroids; with a proof-of-concept that scaffold-free constructs generate cartilaginous repair tissue and can integrate with host tissue when implanted into human osteoarthritic cartilage tissue. These constructs showed promising cartilage repair *in vivo* in rabbits (Grogan et al., 2020). Microspheroids of BMMSC have also been assembled using the Regenova system to produce cartilage and bone-like tissue when cultured in chondrogenic or osteogenic

**FIGURE 14.4**

Inkjet printing with TIPS. (A) A custom printer (TIPS, Hewlett-Packard, Corvallis, OR) with modular print nozzles was used to inkjet droplets directly onto explants of arthritic human cartilage. (B) The printer was used to print layers of cells on electrospun biopaper. (C) Demonstrating feasibility of printing multiple cell types (stained with CFSE or CMTMR, pattern shown in inset). (D) Printing *in situ* into a bovine osteochondral defect.

medium, respectively. Mini-constructs of predifferentiated cartilage and bone when cocultured created a bone-cartilage structure with potential for producing osteochondral tissues (Breathwaite et al., 2019). Porcine ADSC microspheroids were similarly assembled to create cylindrical constructs which were implanted into osteochondral cartilage defects in the trochlear groove of minipigs (Yamasaki et al., 2019). After 6 months, a majority of the defects implanted with these constructs showed new tissue regeneration with positive GAG and collagen type II staining.

Cellular microspheroids and microtissues have also been combined with other 3D-printing approaches. Cells delivered via inkjet printing into preprinted PCL microchambers condensed into microspheroids that matured and fused into cartilaginous tissue (Daly & Kelly, 2019). This process can be scaled up to fabricate osteochondral constructs in a bioreactor. Predifferentiated chondrogenic microtissues have also been inserted in a thermoplastic (PEGT/PBT) polymer scaffold to form cartilage tissue (Mekhileri et al., 2018).

**FIGURE 14.5**

The “Kenzan” approach to scaffold-free bioprinting. (A) An individual microspheroid was generated from 15×10^3 ADSC in one well of a 96 well plate. (B) Microspheroids were placed upon an array of microneedles to form three layers, with each layer containing 31 microspheroids. (C) Overview of a single layer of microspheroids placed on the needle array. (D) Construct removed from the needle after 7 days in culture and implanted into chondral defects (E) created in *ex vivo* human OA cartilage and cultured for 3 weeks. (F, G) Safranin O staining of neocartilaginous tissue showing GAG content and integration with host OA tissue.

14.2.1.4 Freeform Reversible Embedding of Suspended Hydrogels Printing

FRESH involves supporting printed hydrogel inks in thermoreversible slurry of gelatin microparticles (Fig. 14.2E) (Hinton et al., 2015). A similar approach was used to extrude human MSC in a bath of biodegradable and dual-crosslinkable alginate microgels that permitted movement of the print nozzle while providing immediate support for the printed cells that differentiated into

cartilage- or bone-like tissues (Jeon et al., 2019). Fibrinogen bioink containing human MSC spheroids has also been printed into a supporting bath composed of PEG, alginate, and thrombin with encouraging chondrogenic differentiation (de Melo et al., 2020).

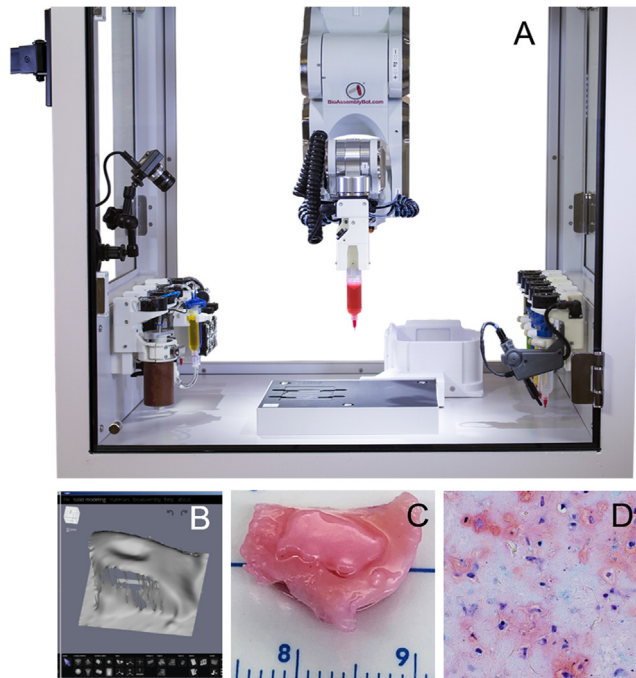
14.2.1.5 Direct In Situ Printing

The vast majority of 3D-printing techniques are directed at engineering tissue *in vitro* with the objective of subsequent implantation into the body. One attractive alternative is printing engineered tissue directly into the human body. Cohen et al. demonstrated proof-of-concept by delivering alginate and demineralized bone matrix via syringe extrusion into surgically created articular defects (Cohen, Lipton, Bonassar, & Lipson, 2010). Although no cells were printed and the femur had been devitalized, this report demonstrated proof-of-concept of the potential of additive manufacturing for direct *in situ* printing.

We provided proof-of-concept for *in situ* 3D printing of live human articular chondrocytes for cartilage repair (Cui, Breitenkamp, Finn, et al., 2012; Cui, Breitenkamp, Lotz, et al., 2012). A modified thermal inkjet printer deposited chondrocytes suspended in photocrosslinkable PEGDA into a cartilage defect created within a bovine osteochondral (cartilage and bone) tissue explant for cartilage repair. This study demonstrated the utility of direct cartilage repair and bioprinting by successfully controlling the placement of individual cells with high viability, by providing a supportive environment for secretion of cartilage ECM protein, by printing material with adequate mechanical properties, and by generating tissue that integrated directly into host tissue. After 6 weeks of culture, we observed that the printed PEG gel and chondrocytes produced cartilaginous neotissue with increased collagen type II (gene expression level), substantial GAG deposition (Safranin O stain), and evidence of histologic integration with host tissue. The compressive modulus of the printed tissue approached 400 kPa, within the range of that reported for articular cartilage (Lai & Levenston, 2010).

We also printed *in situ* into chondral lesions created in human osteoarthritic tissue. We printed human ESC–MSC in GelXA Fibrin (CELLINK) directly into the chondral lesions (Fig. 14.3). Chondrogenic gene expression and neotissue formation were evident following 3 weeks in culture. *In situ* printing into irregular lesions can be challenging with standard three-axis printers. For greater access to the print surface and finer control of printing organic shapes, we used a six-axis bioprinting platform (BioAssemblyBot, Advanced Solutions, Louisville, KY, Fig. 14.6). The BioAssemblyBot uses a robotic arm to control multiple print heads and a laser scanner. The laser scanner generated a 3D image of the surface of the cartilage defect in osteochondral explants obtained from the knee joints of patients undergoing total knee arthroplasty. The 3D model of the defect was used to print ESC–MSC in a fibrin-based bioink directly into the defect. The osteochondral explant was then cultured for 3 weeks and histology showed evidence of cartilage-like tissue that was integrated into the host tissue.

For *in vivo* proof-of-concept, a six-degree-of-freedom robotic arm was used to print a solution of methacrylated HA and acrylate-terminated four-armed PEG in surgical defects created in rabbit femoral trochlea (Ma et al., 2020). A robotic arm was also used to mill a defect in a sheep humerus bone specimen which was then printed with alginate-PEGDA (Lipskas, Deep, & Yao, 2019). In direct contrast to robotic arms, a hand-held “Biopen” was developed to enable manual *in situ* printing of methacrylated gelatin and HA (O’Connell et al., 2016). The Biopen was used to print allogeneic adipose-derived MSC in chondral defects surgically created in sheep femoral condyles (Di Bella et al., 2018).

**FIGURE 14.6**

In situ printing with six-axis robotic arm. (A) The BioAssemblyBot (Advanced Solutions, Lexington, KY) contains a six-axis robotic arm, multiple tools, and various print heads. (B) The integrated laser scanning tool was used to generate a 3D image of a chondral defect in a cartilage explant. (C) A pneumatic print head cartridge extruded bioink into the chondral defect which was cultured for 3 weeks to show (D) neotissue formation (Safranin O stain; magnification 40 $\times$).

14.3 MAJOR CHALLENGES AND PITFALLS

One needs to address the major factors common to most bioprinting approaches which include printability, cell survival, postprint stability and fidelity, biocompatible bioinks and crosslinking mechanisms, appropriate mechanical properties, and the creation of an environment to stimulate neo-cartilage tissue formation. The scientific and technical challenges facing successful bioprinting can be broadly classified into cellular, bioink, and printing technology.

14.3.1 CELL SOURCE

Many cell sources have been investigated for tissue engineering of cartilage. Primary articular chondrocytes are an obvious choice. However, primary human cells are limited in supply and in their capacity to proliferate and create neotissues (Costa, Gonzalez-Garcia, Gomez, & Salmeron-

Sanchez, 2018). While the so-called ACPC have been used in 3D bioprinting (Diloksumpan et al., 2020; Mouser et al., 2020), obtaining these cells for clinical translation remains a challenge. Other progenitor cell sources have been explored for printing cartilage including BMMSC (Levato et al., 2017; Shi et al., 2017), ADSC (Di Bella et al., 2018; Olate-Moya et al., 2020), human umbilical cord blood-derived mesenchymal stem cells (Huang et al., 2021), embryonic-derived MSC (Grogan et al., 2020), and induced pluripotent stem cells (iPSC) (Nguyen et al., 2017). Each of these sources has different advantages and disadvantages with respect to cartilage forming capacity, tissue harvesting morbidity, ethical, and safety concerns (Fisher, Tessaro, Bertocco, Peretti, & Mangiavini, 2017).

The minimal requirements are resilient cells that survive the printing process and cells that maintain the appropriate phenotype and function after printing. Despite the diverse reports on a wide spectrum of cell sources, the optimal cell line has not yet been established. The choice between autologous and allogeneic cell sources is a difficult one. Advantages of allogeneic cells are a well-defined and characterized source of cells with potential for off-the-shelf treatment. Advantages of autologous cells are the absence of immune response with an easier pathway for regulatory approval.

14.3.2 BIOINKS AND SCAFFOLDS

Many challenges have to be overcome before printable scaffolds can be clinically useful. For successful tissue regeneration, the scaffold has to be biocompatible without any biological side effects upon degradation or release of degradation products. At a minimum, the bioink has to rapidly undergo a liquid-to-solid transition to support efficient printing. Traditional methods used in commercial 3D printers, such as extrusion of molten polymer or powder-based printing, are not likely to be compatible with bioprinting for cytotoxic reasons. Approaches for chemical, thermal, ionic, enzymatic, and photocrosslinked stabilization of bioinks are being explored. Other requirements for bioinks for cartilage, meniscus, and bone are properties such as mechanical strength to survive implantation and postoperative mechanical loading, and the appropriate biochemical and biophysical cues to support, induce, and maintain the phenotype of the printed cells.

14.3.3 DELIVERY

The present state-of-the-art includes a variety of delivery systems. At a minimum these delivery methods need to be optimized to deal with the conflicting requirements of spatial resolution, viscosity of bioink, efficiency of printing, and cell density. Maintaining cell suspensions in the print-reservoir to avoid cell clumping during the printing, or worse, clogging of the print nozzles is important. Multiple nozzles may be required for chemical crosslinking of the bioink, for delivery of different cell types or different ingredients in the bioink (multi-ink printing). Finally, a robust platform is needed that can accurately control the spatial location and orientation of the print head, coordinate the delivery of the ink, and maintain the biological integrity of the bioink. Direct *in situ* bioprinting adds even more challenges such as integrating the print system within the operating room and real-time imaging of the print site.

14.4 FUTURE DIRECTIONS

In the 5 years since the previous edition of this book, several advances have been made in identifying cellular sources, exploring biomaterials suitable for bioprinting, and enhancing printing technologies. While progress has been made toward proof-of-concept of translation to animal models, the obstacles to clinical use have yet to be overcome. It is important to demonstrate that 3D printing can recapitulate the crucial architectural features of articular cartilage. Even more important is generating evidence that printed artificial tissue with these appropriate features can resolve the major obstacles facing cartilage tissue engineering, that is, integration with host tissue, long-term durability, and delaying or preventing joint degeneration, and osteoarthritis.

Discoveries in cellular biology, especially in the identification and characterization of the various stages between stem cells and progenitor status are likely to be of great significance in advancing the field of bioprinting. Factors influencing cell differentiation including biochemical, biomechanical, and other biophysical cues are critical in determining the fate of printed tissue, especially in multiphasic tissues.

Research in biomaterials is required to enhance printing efficiency and material properties. Other areas worthy of attention are responsive biomaterials that release growth factors or agents that modulate proliferation and other cellular functions such as differentiation and matrix production. The ultimate goal is not merely to engineer biomimetic features, but to generate tissue that survives physiologic loading, remodels to adapt to changing conditions, and self-heals when exposed to injury.

Finally, while *in vivo* models are essential for clinical translation, these experiments are typically too expensive or time-consuming for the exploratory stage. Therefore more time- and cost-efficient *in vitro* or *ex vivo* models are necessary and, more importantly, need to be validated with clinical results.

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References

- Abbadessa, A., Mouser, V. H. M., Blokzijl, M. M., Gawlitta, D., Dhert, W. J. A., Hennink, W. E., ... Vermonden, T. (2016). A synthetic thermosensitive hydrogel for cartilage bioprinting and its biofunctionalization with polysaccharides. *Biomacromolecules*, 17(6), 2137–2147.
- Alsalameh, S., Amin, R., Gemba, T., & Lotz, M. (2004). Identification of mesenchymal progenitor cells in normal and osteoarthritic human articular cartilage. *Arthritis and Rheumatism*, 50(5), 1522–1532.
- Antich, C., de Vicente, J., Jimenez, G., Chocarro, C., Carrillo, E., Montanez, E., ... Marchal, J. A. (2020). Bio-inspired hydrogel composed of hyaluronic acid and alginate as a potential bioink for 3D bioprinting of articular cartilage engineering constructs. *Acta Biomaterialia*, 106, 114–123.

- Asari, A., Miyauchi, S., Kuriyama, S., Machida, A., Kohno, K., & Uchiyama, Y. (1994). Localization of hyaluronic acid in human articular cartilage. *The Journal of Histochemistry and Cytochemistry: Official Journal of the Histochemistry Society*, 42(4), 513–522.
- Aydelotte, M. B., Greenhill, R. R., & Kuettner, K. E. (1988). Differences between sub-populations of cultured bovine articular chondrocytes. II. Proteoglycan metabolism. *Connective Tissue Research*, 18(3), 223–234.
- Aydelotte, M. B., & Kuettner, K. E. (1988). Differences between sub-populations of cultured bovine articular chondrocytes. I. Morphology and cartilage matrix production. *Connective Tissue Research*, 18(3), 205–222.
- Bayliss, M. T., Venn, M., Maroudas, A., & Ali, S. Y. (1983). Structure of proteoglycans from different layers of human articular cartilage. *The Biochemical Journal*, 209(2), 387–400.
- Beke, S., Anjum, F., Tsushima, H., Ceseracciu, L., Chieragatti, E., Diaspro, A., . . . Brandi, F. (2012). Towards excimer-laser-based stereolithography: A rapid process to fabricate rigid biodegradable photopolymer scaffolds. *Journal of the Royal Society, Interface/the Royal Society*, 9(76), 3017–3026.
- Benninghoff, A. (1925). Form und bau der Geleknorpel in ihren Beziehungen zur Funktion. *Zeitschrift für Zellforschung und Mikroskopische Anatomie (Vienna, Austria: 1948)*, 2, 783–825.
- Billiet, T., Vandenhaute, M., Schelfhout, J., Van Vlierberghe, S., & Dubrue, P. (2012). A review of trends and limitations in hydrogel-rapid prototyping for tissue engineering. *Biomaterials*, 33(26), 6020–6041.
- Breathwaite, E. K., Weaver, J. R., Murchison, A. C., Treadwell, M. L., Odanga, J. J., & Lee, J. B. (2019). Scaffold-free bioprinted osteogenic and chondrogenic systems to model osteochondral physiology. *Biomedical Materials (Bristol, England)*, 14(6), 065010.
- Brenckle, M. A., Tao, H., Kim, S., Paquette, M., Kaplan, D. L., & Omenetto, F. G. (2013). Protein-protein nanoimprinting of silk fibroin films. *Advanced Materials*, 25(17), 2409–2414.
- Brittberg, M., Lindahl, A., Nilsson, A., Ohlsson, C., Isaksson, O., & Peterson, L. (1994). Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *The New England Journal of Medicine*, 331(14), 889–895.
- Brittberg, M., Recker, D., Ilgenfritz, J., Saris, D. B. F., & Group, S. E. S. (2018). Matrix-applied characterized autologous cultured chondrocytes vs microfracture: Five-year follow-up of a prospective randomized trial. *The American Journal of Sports Medicine*, 46(6), 1343–1351.
- Buckley, M. R., Gleghorn, J. P., Bonassar, L. J., & Cohen, I. (2008). Mapping the depth dependence of shear properties in articular cartilage. *Journal of Biomechanics*, 41(11), 2430–2437.
- Buckwalter, J. A., & Mankin, H. J. (1998). Articular cartilage: Tissue design and chondrocyte-matrix interactions. *Instructional Course Lectures*, 47, 477–486.
- Bulman, S. E., Barron, V., Coleman, C. M., & Barry, F. (2013). Enhancing the mesenchymal stem cell therapeutic response: Cell localization and support for cartilage repair. *Tissue Engineering. Part B, Reviews*, 19(1), 58–68.
- Burr, D. B. (2004). “Anatomy and physiology of the mineralized tissues: Role in the pathogenesis of osteoarthritis. *Osteoarthritis and Cartilage/OARS, Osteoarthritis Research Society*, 12(Suppl A), S20–S30.
- Buschmann, M. D., Maurer, A. M., Berger, E., Perumbuli, P., & Hunziker, E. B. (2000). Ruthenium hexaammine trichloride chemography for aggrecan mapping in cartilage is a sensitive indicator of matrix degradation. *The Journal of Histochemistry and Cytochemistry: Official Journal of the Histochemistry Society*, 48(1), 81–88.
- Carletti, E., Motta, A., & Migliaresi, C. (2011). Scaffolds for tissue engineering and 3D cell culture. *Methods in Molecular Biology*, 695, 17–39.
- Chahal, D., Ahmadi, A., & Cheung, K. C. (2012). Improving piezoelectric cell printing accuracy and reliability through neutral buoyancy of suspensions. *Biotechnology and Bioengineering*, 109(11), 2932–2940.
- Clar, C., Cummins, E., McIntyre, L., Thomas, S., Lamb, J., Bain, L., . . . Waugh, N. (2005). Clinical and cost-effectiveness of autologous chondrocyte implantation for cartilage defects in knee joints: Systematic review and economic evaluation. *Health Technology Assessment (Winchester, England)*, 9(47), 1–82, iii–iv, ix–x.
- Cohen, D. L., Lipton, J. I., Bonassar, L. J., & Lipton, H. (2010). Additive manufacturing for in situ repair of osteochondral defects. *Biofabrication*, 2(3), 035004.

- Costa, E., Gonzalez-Garcia, C., Gomez Ribelles, J. L., & Salmeron-Sanchez, M. (2018). Maintenance of chondrocyte phenotype during expansion on PLLA microtopographies. *Journal of Tissue Engineering*, 9, 2041731418789829.
- Costantini, M., Idaszek, J., Szoke, K., Jaroszewicz, J., Dentini, M., Barbetta, A., . . . Swieszkowski, W. (2016). 3D bioprinting of BM-MSCs-loaded ECM biomimetic hydrogels for in vitro neocartilage formation. *Biofabrication*, 8(3), 035002.
- Crawford, D. C., DeBerardino, T. M., & Williams, R. J., 3rd (2012). NeoCart, an autologous cartilage tissue implant, compared with microfracture for treatment of distal femoral cartilage lesions: An FDA phase-II prospective, randomized clinical trial after two years. *The Journal of Bone and Joint Surgery. American Volume*, 94(11), 979–989.
- Critchley, S., Sheehy, E. J., Cunniffe, G., Diaz-Payno, P., Carroll, S. F., Jeon, O., . . . Kelly, D. J. (2020). 3D printing of fibre-reinforced cartilaginous templates for the regeneration of osteochondral defects. *Acta Biomaterialia*, 113, 130–143.
- Cui, X., Breitenkamp, K., Finn, M. G., Lotz, M., & D’Lima, D. D. (2012). Direct human cartilage repair using three-dimensional bioprinting technology. *Tissue Engineering. Part A*, 18(11–12), 1304–1312.
- Cui, X., Breitenkamp, K., Lotz, M., & D’Lima, D. (2012). Synergistic action of fibroblast growth factor-2 and transforming growth factor-beta1 enhances bioprinted human neocartilage formation. *Biotechnology and Bioengineering*, 109(9), 2357–2368.
- Cui, X., Boland, T., D’Lima, D. D., & Lotz, M. K. (2012). Thermal inkjet printing in tissue engineering and regenerative medicine. *Recent Patents on Drug Delivery & Formulation*, 6(2), 149–155.
- Daly, A. C., Critchley, S. E., Rencsok, E. M., & Kelly, D. J. (2016). A comparison of different bioinks for 3D bioprinting of fibrocartilage and hyaline cartilage. *Biofabrication*, 8(4), 045002.
- Daly, A. C., & Kelly, D. J. (2019). Biofabrication of spatially organised tissues by directing the growth of cellular spheroids within 3D printed polymeric microchambers. *Biomaterials*, 197, 194–206.
- Darling, E. M., Hu, J. C., & Athanasiou, K. A. (2004). Zonal and topographical differences in articular cartilage gene expression. *Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society*, 22(6), 1182–1187.
- de Melo, B. A. G., Jodat, Y. A., Cruz, E. M., Benincasa, J. C., Shin, S. R., & Porcionatto, M. A. (2020). Strategies to use fibrinogen as bioink for 3D bioprinting fibrin-based soft and hard tissues. *Acta Biomaterialia*, 117, 60–76.
- de Ruijter, M., Ribeiro, A., Dokter, I., Castilho, M., & Malda, J. (2019). Simultaneous micropatterning of fibrous meshes and bioinks for the fabrication of living tissue constructs. *Advanced Healthcare Materials*, 8(7), e1800418.
- Devitt, B. M., Bell, S. W., Webster, K. E., Feller, J. A., & Whitehead, T. S. (2017). Surgical treatments of cartilage defects of the knee: Systematic review of randomised controlled trials. *The Knee*, 24(3), 508–517.
- Di Bella, C., Duchi, S., O’Connell, C. D., Blanchard, R., Augustine, C., Yue, Z., . . . Choong, P. F. (2018). In situ handheld three-dimensional bioprinting for cartilage regeneration. *Journal of Tissue Engineering and Regenerative Medicine*, 12(3), 611–621.
- DiCesare, P. E., Morgelin, M., Carlson, C. S., Pasumarti, S., & Paulsson, M. (1995). Cartilage oligomeric matrix protein: Isolation and characterization from human articular cartilage. *Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society*, 13(3), 422–428.
- Diloksumpan, P., de Ruijter, M., Castilho, M., Gbureck, U., Vermonden, T., van Weeren, P. R., . . . Levato, R. (2020). Combining multi-scale 3D printing technologies to engineer reinforced hydrogel-ceramic interfaces. *Biofabrication*, 12(2), 025014.
- Dowthwaite, G. P., Bishop, J. C., Redman, S. N., Khan, I. M., Rooney, P., Evans, D. J., . . . Archer, C. W. (2004). The surface of articular cartilage contains a progenitor cell population. *Journal of Cell Science*, 117(Pt 6), 889–897.

- Dudhia, J. (2005). Aggrecan, aging and assembly in articular cartilage. *Cellular and Molecular Life Sciences: CMLS*, 62(19–20), 2241–2256.
- Fedorovich, N. E., Schuurman, W., Wijnberg, H. M., Prins, H. J., van Weeren, P. R., Malda, J., ... Dhert, W. J. (2012). Biofabrication of osteochondral tissue equivalents by printing topologically defined, cell-laden hydrogel scaffolds. *Tissue Engineering. Part C, Methods*, 18(1), 33–44.
- Filardo, G., Madry, H., Jelic, M., Roffi, A., Cucchiari, M., & Kon, E. (2013). Mesenchymal stem cells for the treatment of cartilage lesions: From preclinical findings to clinical application in orthopaedics. *Knee Surgery, Sports Traumatology, Arthroscopy: Official Journal of the ESSKA*, 21(8), 1717–1729.
- Fisher, J. N., Tessaro, I., Bertocco, T., Peretti, G. M., & Mangiavini, L. (2017). The application of stem cells from different tissues to cartilage repair. *Stem Cells International*, 2017, 2761678.
- Flannery, C. R., Hughes, C. E., Schumacher, B. L., Tudor, D., Aydelotte, M. B., Kuettner, K. E., & Caterson, B. (1999). Articular cartilage superficial zone protein (SZP) is homologous to megakaryocyte stimulating factor precursor and is a multifunctional proteoglycan with potential growth-promoting, cytoprotective, and lubricating properties in cartilage metabolism. *Biochemical and Biophysical Research Communications*, 254(3), 535–541.
- Franzen, A., Inerot, S., Hejderup, S. O., & Heinegard, D. (1981). Variations in the composition of bovine hip articular cartilage with distance from the articular surface. *The Biochemical Journal*, 195(3), 535–543.
- Gao, J., Ding, X., Yu, X., Chen, X., Zhang, X., Cui, S., ... Ding, J. (2021). Cell-free bilayered porous scaffolds for osteochondral regeneration fabricated by continuous 3D-printing using nascent physical hydrogel as ink. *Advanced Healthcare Materials*, 10(3), e2001404.
- Gortz, S., & Bugbee, W. D. (2006). Allografts in articular cartilage repair. *The Journal of Bone and Joint Surgery. American Volume*, 88(6), 1374–1384.
- Goyal, D., Keyhani, S., Goyal, A., Lee, E. H., Hui, J. H., & Vaziri, A. S. (2014). Evidence-based status of osteochondral cylinder transfer techniques: A systematic review of level I and II studies. *Arthroscopy: The Journal of Arthroscopic & Related Surgery: Official Publication of the Arthroscopy Association of North America and the International Arthroscopy Association*, 30(4), 497–505.
- Goyal, D., Keyhani, S., Lee, E. H., & Hui, J. H. (2013). Evidence-based status of microfracture technique: A systematic review of level I and II studies. *Arthroscopy: The Journal of Arthroscopic & Related Surgery: Official Publication of the Arthroscopy Association of North America and the International Arthroscopy Association*, 29(9), 1579–1588.
- Graham, A. D., Olof, S. N., Burke, M. J., Armstrong, J. P. K., Mikhailova, E. A., Nicholson, J. G., ... Bayley, H. (2017). High-resolution patterned cellular constructs by droplet-based 3D printing. *Scientific Reports*, 7(1), 7004.
- Grayson, C. W., & Decker, R. C. (2012). Total joint arthroplasty for persons with osteoarthritis. *PM & R*, 4(5 Suppl), S97–S103.
- Grogan, S. P., Chung, P. H., Soman, P., Chen, P., Lotz, M. K., Chen, S., & D'Lima, D. D. (2013). Digital micromirror device projection printing system for meniscus tissue engineering. *Acta Biomaterialia*, 9(7), 7218–7226.
- Grogan, S. P., Dorthe, E. W., Glembotski, N. E., Gaul, F., & D'Lima, D. D. (2020). Cartilage tissue engineering combining microspheroid building blocks and microneedle arrays. *Connective Tissue Research*, 61(2), 229–243.
- Grogan, S. P., Duffy, S. F., Pauli, C., Koziol, J. A., Su, A. I., D'Lima, D. D., & Lotz, M. K. (2013). Zone-specific gene expression patterns in articular cartilage. *Arthritis and Rheumatism*, 65(2), 418–428.
- Grogan, S. P., Miyaki, S., Asahara, H., D'Lima, D. D., & Lotz, M. K. (2009). Mesenchymal progenitor cell markers in human articular cartilage: Normal distribution and changes in osteoarthritis. *Arthritis Research & Therapy*, 11(3), R85.

- Henrionnet, C., Pourchet, L., Neybecker, P., Messaoudi, O., Gillet, P., Loeuille, D., ... Pinzano, A. (2020). Combining innovative bioink and low cell density for the production of 3D-bioprinted cartilage substitutes: A pilot study. *Stem Cells International*, 2020, 2487072.
- Hidaka, C., Cheng, C., Alexandre, D., Bhargava, M., & Torzilli, P. A. (2006). Maturation differences in superficial and deep zone articular chondrocytes. *Cell and Tissue Research*, 323(1), 127–135.
- Hinton, T. J., Jallerat, Q., Palchesko, R. N., Park, J. H., Grodzicki, M. S., Shue, H. J., ... Feinberg, A. W. (2015). Three-dimensional printing of complex biological structures by freeform reversible embedding of suspended hydrogels. *Science Advances*, 1(9), e1500758.
- Hoemann, C. D., Lafantaisie-Favreau, C. H., Lascau-Coman, V., Chen, G., & Guzman-Morales, J. (2012). The cartilage-bone interface. *The Journal of Knee Surgery*, 25(2), 85–97.
- Holmes, B., Zhu, W., Li, J., Lee, J. D., & Zhang, L. G. (2015). Development of novel three-dimensional printed scaffolds for osteochondral regeneration. *Tissue Engineering. Part A*, 21(1–2), 403–415.
- Huang, J., Huang, Z., Liang, Y., Yuan, W., Bian, L., Duan, L., ... Xia, J. (2021). 3D printed gelatin/hydroxyapatite scaffolds for stem cell chondrogenic differentiation and articular cartilage repair. *Biomaterials Science*, 9(7), 2620–2630.
- Hunziker, E. B., Michel, M., & Studer, D. (1997). Ultrastructure of adult human articular cartilage matrix after cryotechnical processing. *Microscopy Research and Technique*, 37(4), 271–284.
- Hunziker, E. B., Quinn, T. M., & Hauselmann, H. J. (2002). Quantitative structural organization of normal adult human articular cartilage. *Osteoarthritis and Cartilage/OARS, Osteoarthritis Research Society*, 10(7), 564–572.
- Jacoby, R. K., & Jayson, M. I. (1975). Proceedings: Adult human articular cartilage in organ culture. Synthesis of glycosaminoglycan, effect of hyperoxia, and zonal variation of matrix synthesis. *Annals of the Rheumatic Diseases*, 34(5), 468.
- Jay, G. D., Haberstroh, K., & Cha, C. J. (1998). Comparison of the boundary-lubricating ability of bovine synovial fluid, lubricin, and Healon. *Journal of Biomedical Materials Research*, 40(3), 414–418.
- Jeon, O., Lee, Y. B., Jeong, H., Lee, S. J., Wells, D., & Alsberg, E. (2019). Individual cell-only bioink and photocurable supporting medium for 3D printing and generation of engineered tissues with complex geometries. *Materials Horizons*, 6(8), 1625–1631.
- Khan, I. M., Gilbert, S. J., Singhrao, S. K., Duance, V. C., & Archer, C. W. (2008). Cartilage integration: Evaluation of the reasons for failure of integration during cartilage repair. A review. *European Cells & Materials*, 16, 26–39.
- Khan, I. M., Salter, D. M., Bayliss, M. T., Thomson, B. M., & Archer, C. W. (2001). Expression of clusterin in the superficial zone of bovine articular cartilage. *Arthritis and Rheumatism*, 44(8), 1795–1799.
- Kizawa, H., Kou, I., Iida, A., Sudo, A., Miyamoto, Y., Fukuda, A., ... Ikegawa, S. (2005). An aspartic acid repeat polymorphism in asporin inhibits chondrogenesis and increases susceptibility to osteoarthritis. *Nature Genetics*, 37(2), 138–144.
- Klein, T. J., Rizzi, S. C., Reichert, J. C., Georgi, N., Malda, J., Schuurman, W., ... Hutmacher, D. W. (2009). Strategies for zonal cartilage repair using hydrogels. *Macromolecular Bioscience*, 9(11), 1049–1058.
- Kon, E., Filardo, G., Di Martino, A., & Marcacci, M. (2012). ACI and MACI. *The Journal of Knee Surgery*, 25(1), 17–22.
- Lai, J. H., & Levenston, M. E. (2010). Meniscus and cartilage exhibit distinct intra-tissue strain distributions under unconfined compression. *Osteoarthritis and Cartilage/OARS, Osteoarthritis Research Society*, 18(10), 1291–1299.
- Levato, R., Webb, W. R., Otto, I. A., Mensinga, A., Zhang, Y., van Rijen, M., van Weeren, R., Khan, I. M., & Malda, J. (2017). The bio in the ink: Cartilage regeneration with bioprintable hydrogels and articular cartilage-derived progenitor cells. *Acta Biomaterialia*, 61, 41–53.

- Li, Z., Zhang, X., Yuan, T., Zhang, Y., Luo, C., Zhang, J., Liu, Y., & Fan, W. (2020). Addition of platelet-rich plasma to silk fibroin hydrogel bioprinting for cartilage regeneration. *Tissue Engineering. Part A*, 26 (15–16), 886–895.
- Lin, H., Zhang, D., Alexander, P. G., Yang, G., Tan, J., Cheng, A. W., & Tuan, R. S. (2013). Application of visible light-based projection stereolithography for live cell-scaffold fabrication with designed architecture. *Biomaterials*, 34(2), 331–339.
- Lipskas, J., Deep, K., & Yao, W. (2019). Robotic-assisted 3D bio-printing for repairing bone and cartilage defects through a minimally invasive approach. *Scientific Reports*, 9(1), 3746.
- Lorenzo, P., Bayliss, M. T., & Heinegard, D. (1998). A novel cartilage protein (CILP) present in the mid-zone of human articular cartilage increases with age. *The Journal of Biological Chemistry*, 273(36), 23463–23468.
- Lu, L., Yuan, S., Wang, J., Shen, Y., Deng, S., Xie, L., & Yang, Q. (2018). The formation mechanism of hydrogels. *Current Stem Cell Research & Therapy*, 13(7), 490–496.
- Ma, K., Zhao, T., Yang, L., Wang, P., Jin, J., Teng, H., Xia, D., Zhu, L., Li, L., Jiang, Q., & Wang, X. (2020). Application of robotic-assisted in situ 3D printing in cartilage regeneration with HAMA hydrogel: An in vivo study. *Journal of Advanced Research*, 23, 123–132.
- Malda, J., ten Hoope, W., Schuurman, W., van Osch, G. J., van Weeren, P. R., & Dhert, W. J. (2010). Localization of the potential zonal marker clusterin in native cartilage and in tissue-engineered constructs. *Tissue Engineering. Part A*, 16(3), 897–904.
- Marcacci, M., Kon, E., Delcogliano, M., Filardo, G., Busacca, M., & Zaffagnini, S. (2007). Arthroscopic autologous osteochondral grafting for cartilage defects of the knee: Prospective study results at a minimum 7-year follow-up. *The American Journal of Sports Medicine*, 35(12), 2014–2021.
- Markstedt, K., Mantas, A., Tournier, I., Martinez Avila, H., Hagg, D., & Gatenholm, P. (2015). 3D bioprinting human chondrocytes with nanocellulose-alginate bioink for cartilage tissue engineering applications. *Biomacromolecules*, 16(5), 1489–1496.
- Maroudas, A., Muir, H., & Wingham, J. (1969). The correlation of fixed negative charge with glycosaminoglycan content of human articular cartilage. *Biochimica et Biophysica Acta*, 177(3), 492–500.
- Mekhileri, N. V., Lim, K. S., Brown, G. C. J., Mutreja, I., Schon, B. S., Hooper, G. J., & Woodfield, T. B. F. (2018). Automated 3D bioassembly of micro-tissues for biofabrication of hybrid tissue engineered constructs. *Biofabrication*, 10(2), 024103.
- Melchels, F. P. W., Dhert, W. J. A., Hutmacher, D. W., & Malda, J. (2014). Development and characterisation of a new bioink for additive tissue manufacturing. *Journal of Materials Chemistry B*, 2(16), 2282–2289.
- Melchels, F. P. W., Domingos, M. A. N., Klein, T. J., Malda, J., Bartolo, P. J., & Hutmacher, D. W. (2012). Additive manufacturing of tissue and organs. *Progress in Polymer Science*, 37, 1079–1104.
- Miosge, N., Flachsbarth, K., Goetz, W., Schultz, W., Kresse, H., & Herken, R. (1994). Light and electron microscopical immunohistochemical localization of the small proteoglycan core proteins decorin and biglycan in human knee joint cartilage. *The Histochemical Journal*, 26(12), 939–945.
- Mironov, V., Visconti, R. P., Kasyanov, V., Forgacs, G., Drake, C. J., & Markwald, R. R. (2009). Organ printing: Tissue spheroids as building blocks. *Biomaterials*, 30(12), 2164–2174.
- Mithoefer, K., Williams, R. J., 3rd, Warren, R. F., Potter, H. G., Spock, C. R., Jones, E. C., . . . Marx, R. G. (2005). The microfracture technique for the treatment of articular cartilage lesions in the knee. A prospective cohort study. *The Journal of Bone and Joint Surgery. American Volume*, 87(9), 1911–1920.
- Mitrovic, D. R., & Darmon, N. (1994). Characterization of proteoglycans synthesized by different layers of adult human femoral head cartilage. *Osteoarthritis and Cartilage/OARS, Osteoarthritis Research Society*, 2 (2), 119–131.

- Mohanraj, B., Farran, A. J., Mauck, R. L., & Dodge, G. R. (2013). Time-dependent functional maturation of scaffold-free cartilage tissue analogs. *Journal of Biomechanics*, 47(9), 2137–2142.
- Moldovan, N. I., Hibino, N., & Nakayama, K. (2017). Principles of the kenzan method for robotic cell spheroid-based three-dimensional bioprinting. *Tissue Engineering. Part B, Reviews*, 23(3), 237–244.
- Moroni, L., Hamann, D., Paoluzzi, L., Pieper, J., de Wijn, J. R., & van Blitterswijk, C. A. (2008). Regenerating articular tissue by converging technologies. *PLoS One*, 3(8), e3032.
- Mouser, V. H. M., Levato, R., Mensinga, A., Dhert, W. J. A., Gawlitta, D., & Malda, J. (2020). Bio-ink development for three-dimensional bioprinting of hetero-cellular cartilage constructs. *Connective Tissue Research*, 61(2), 137–151.
- Mouser, V. H. M., Melchels, F. P., Visser, J., Dhert, W. J., Gawlitta, D., & Malda, J. (2016). Yield stress determines bioprintability of hydrogels based on gelatin-methacryloyl and gellan gum for cartilage bioprinting. *Biofabrication*, 8(3), 035003.
- Mow, V. C., Proctor, C. S., & Kelly, M. A. (2012). Biomechanics of articular cartilage. In M. Nordin, & V. H. Frankel (Eds.), *Basic biomechanics of the musculoskeletal system*. Philadelphia: Lippincott Williams & Wilkins.
- Moxon, S. R., Ferreira, M. J. S., Santos, P. D., Popa, B., Gloria, A., Katsarava, R., ... Domingos, M. A. N. (2020). A preliminary evaluation of the pro-chondrogenic potential of 3D-bioprinted poly(ester urea) scaffolds. *Polymers (Basel)*, 12(7), 1478.
- Muir, H., Bullough, P., & Maroudas, A. (1970). The distribution of collagen in human articular cartilage with some of its physiological implications. *The Journal of Bone and Joint Surgery. British Volume*, 52(3), 554–563.
- Muller, M., Fisch, P., Molnar, M., Eggert, S., Binelli, M., Maniura-Weber, K., & Zenobi-Wong, M. (2020). Development and thorough characterization of the processing steps of an ink for 3D printing for bone tissue engineering. *Materials Science Engineering: C Materials for Biological Applications*, 108, 110510.
- Muller, M., Ozturk, E., Arlov, O., Gatenholm, P., & Zenobi-Wong, M. (2017). Alginate sulfate-nanocellulose bioinks for cartilage bioprinting applications. *Annals of Biomedical Engineering*, 45(1), 210–223.
- Murray, R. C., Smith, R. K., Henson, F. M., & Goodship, A. (2001). The distribution of cartilage oligomeric matrix protein (COMP) in equine carpal articular cartilage and its variation with exercise and cartilage deterioration. *Veterinary Journal (London, England: 1997)*, 162(2), 121–128.
- Nguyen, D., Hagg, D. A., Forsman, A., Ekholm, J., Nimkingratana, P., Brantsing, C., ... Simonsson, S. (2017). Cartilage tissue engineering by the 3D bioprinting of iPS cells in a nanocellulose/alginate bioink. *Scientific Reports*, 7(1), 658.
- Noble, P. C., Gordon, M. J., Weiss, J. M., Reddix, R. N., Conditt, M. A., & Mathis, K. B. (2005). Does total knee replacement restore normal knee function? *Clinical Orthopaedics and Related Research*, 431, 157–165.
- Norotte, C., Marga, F. S., Niklason, L. E., & Forgacs, G. (2009). Scaffold-free vascular tissue engineering using bioprinting. *Biomaterials*, 30(30), 5910–5917.
- O'Connell, C. D., Di Bella, C., Thompson, F., Augustine, C., Beirne, S., Cornock, R., ... Wallace, G. G. (2016). Development of the biopen: A handheld device for surgical printing of adipose stem cells at a chondral wound site. *Biofabrication*, 8(1), 015019.
- Olate-Moya, F., Arens, L., Wilhelm, M., Mateos-Timoneda, M. A., Engel, E., & Palza, H. (2020). Chondroinductive alginate-based hydrogels having graphene oxide for 3D printed scaffold fabrication. *ACS Applied Materials & Interfaces*, 12(4), 4343–4357.
- Olee, T., Grogan, S. P., Lotz, M. K., Colwell, C. W., Jr., D'Lima, D. D., & Snyder, E. Y. (2014). Repair of cartilage defects in arthritic tissue with differentiated human embryonic stem cells. *Tissue Engineering. Part A*, 20(3–4), 683–692.

- Orth, P., Cucchiari, M., Kohn, D., & Madry, H. (2013). Alterations of the subchondral bone in osteochondral repair—translational data and clinical evidence. *European Cells & Materials*, 25, 299–316, discussion 314–296.
- Pabbruwe, M. B., Esfandiari, E., Kafienah, W., Tarlton, J. F., & Hollander, A. P. (2009). Induction of cartilage integration by a chondrocyte/collagen-scaffold implant. *Biomaterials*, 30(26), 4277–4286.
- Peiffer, Q. C., de Ruijter, M., van Duijn, J., Crottet, D., Dominic, E., Malda, J., & Castilho, M. (2020). Melt electrowriting onto anatomically relevant biodegradable substrates: Resurfacing a diarthrodial joint. *Materials & Design*, 195, 109025.
- Pelletier, J. P., Jovanovic, D. V., Lascau-Coman, V., Fernandes, J. C., Manning, P. T., Connor, J. R., ... Martel-Pelletier, J. (2000). Selective inhibition of inducible nitric oxide synthase reduces progression of experimental osteoarthritis in vivo: Possible link with the reduction in chondrocyte apoptosis and caspase 3 level. *Arthritis and Rheumatism*, 43(6), 1290–1299.
- Pfister, B. E., Aydelotte, M. B., Burkhart, W., Kuettner, K. E., & Schmid, T. M. (2001). Del1: A new protein in the superficial layer of articular cartilage. *Biochemical and Biophysical Research Communications*, 286(2), 268–273.
- Piras, C. C., & Smith, D. K. (2020). Multicomponent polysaccharide alginate-based bioinks. *Journal of Materials Chemistry B*, 8(36), 8171–8188.
- Poole, A. R., Rosenberg, L. C., Reiner, A., Ionescu, M., Bogoch, E., & Roughley, P. J. (1996). Contents and distributions of the proteoglycans decorin and biglycan in normal and osteoarthritic human articular cartilage. *Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society*, 14(5), 681–689.
- Pullig, O., Weseloh, G., Ronneberger, D., Kakonen, S., & Swoboda, B. (2000). Chondrocyte differentiation in human osteoarthritis: Expression of osteocalcin in normal and osteoarthritic cartilage and bone. *Calcified Tissue International*, 67(3), 230–240.
- Rasanen, P., Paavolainen, P., Sintonen, H., Koivisto, A. M., Blom, M., Ryyanen, O. P., & Roine, R. P. (2007). Effectiveness of hip or knee replacement surgery in terms of quality-adjusted life years and costs. *Acta Orthopaedica*, 78(1), 108–115.
- Ratcliffe, A., Fryer, P. R., & Hardingham, T. E. (1984). The distribution of aggregating proteoglycans in articular cartilage: Comparison of quantitative immunoelectron microscopy with radioimmunoassay and biochemical analysis. *The Journal of Histochemistry and Cytochemistry: Official Journal of the Histochemistry Society*, 32(2), 193–201.
- Roelofs, A. J., Rocke, J. P., & De Bari, C. (2013). Cell-based approaches to joint surface repair: A research perspective. *Osteoarthritis and Cartilage/OARS, Osteoarthritis Research Society*, 21(7), 892–900.
- Roughley, P. J. (2006). The structure and function of cartilage proteoglycans. *European Cells & Materials*, 12, 92–101.
- Sakai, S., Ueda, K., Gantumur, E., Taya, M., & Nakamura, M. (2018). Drop-on-drop multimaterial 3D bio-printing realized by peroxidase-mediated cross-linking. *Macromolecular Rapid Communications*, 39, 3. Available from <https://doi.org/10.1002/marc.201700534>.
- Saunders, R. E., Gough, J. E., & Derby, B. (2008). Delivery of human fibroblast cells by piezoelectric drop-on-demand inkjet printing. *Biomaterials*, 29(2), 193–203.
- Schinagl, R. M., Gurskis, D., Chen, A. C., & Sah, R. L. (1997). Depth-dependent confined compression modulus of full-thickness bovine articular cartilage. *Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society*, 15(4), 499–506.
- Schnapper, A., & Meyer, W. (2004). Osteopontin distribution in the canine skeleton during growth and structural maturation. *Cells, Tissues, Organs*, 178(3), 158–167.
- Schumacher, B. L., Hughes, C. E., Kuettner, K. E., Caterson, B., & Aydelotte, M. B. (1999). Immunodetection and partial cDNA sequence of the proteoglycan, superficial zone protein, synthesized by cells lining synovial joints. *Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society*, 17(1), 110–120.

- Schuurman, W., Harimulyo, E. B., Gawlitta, D., Woodfield, T. B., Dhert, W. J., van Weeren, P. R., & Malda, J. (2013). Three-dimensional assembly of tissue-engineered cartilage constructs results in cartilaginous tissue formation without retention of zonal characteristics. *Journal of Tissue Engineering and Regenerative Medicine*, 10(4), 315–324.
- Schuurman, W., Levett, P. A., Pot, M. W., van Weeren, P. R., Dhert, W. J., Hutmacher, D. W., ... Malda, J. (2013). Gelatin-methacrylamide hydrogels as potential biomaterials for fabrication of tissue-engineered cartilage constructs. *Macromolecular Bioscience*, 13(5), 551–561.
- Sherman, S. L., Garrity, J., Bauer, K., Cook, J., Stannard, J., & Bugbee, W. (2014). Fresh osteochondral allograft transplantation for the knee: Current concepts. *The Journal of the American Academy of Orthopaedic Surgeons*, 22(2), 121–133.
- Sherwood, J. K., Riley, S. L., Palazzolo, R., Brown, S. C., Monkhouse, D. C., Coates, M., ... Ratcliffe, A. (2002). A three-dimensional osteochondral composite scaffold for articular cartilage repair. *Biomaterials*, 23(24), 4739–4751.
- Shi, W., Sun, M., Hu, X., Ren, B., Cheng, J., Li, C., ... Ao, Y. (2017). Structurally and functionally optimized silk-fibroin-gelatin scaffold using 3D printing to repair cartilage injury in vitro and in vivo. *Advanced Materials*, 29, 29. Available from <https://doi.org/10.1002/adma.201701089>.
- Shim, J. H., Jang, K. M., Hahn, S. K., Park, J. Y., Jung, H., Oh, K., ... Cho, D. W. (2016). Three-dimensional bioprinting of multilayered constructs containing human mesenchymal stromal cells for osteochondral tissue regeneration in the rabbit knee joint. *Biofabrication*, 8(1), 014102.
- Siczkowski, M., & Watt, F. M. (1990). Subpopulations of chondrocytes from different zones of pig articular cartilage. Isolation, growth and proteoglycan synthesis in culture. *Journal of Cell Science*, 97(Pt 2), 349–360.
- Soman, P., Chung, P. H., Zhang, A. P., & Chen, S. (2013). Digital microfabrication of user-defined 3D microstructures in cell-laden hydrogels. *Biotechnology and Bioengineering*, 110(11), 3038–3047.
- Spiller, K. L., Maher, S. A., & Lowman, A. M. (2011). Hydrogels for the repair of articular cartilage defects. *Tissue Engineering. Part B, Reviews*, 17(4), 281–299.
- Stasiak, J. W. (2017). *Industrial applications of 3D inkjet printing in the life sciences. Handbook of industrial inkjet printing: A full system approach* (pp. 661–680). Wiley VCH.
- Sun, Y., You, Y., Jiang, W., Zhai, Z., & Dai, K. (2019). 3D-bioprinting a genetically inspired cartilage scaffold with GDF5-conjugated BMSC-laden hydrogel and polymer for cartilage repair. *Theranostics*, 9(23), 6949–6961.
- Theodoropoulos, J. S., De Croos, J. N., Park, S. S., Pilliar, R., & Kandel, R. A. (2011). Integration of tissue-engineered cartilage with host cartilage: An in vitro model. *Clinical Orthopaedics and Related Research*, 469(10), 2785–2795.
- Tirella, A., Vozzi, F., De Maria, C., Vozzi, G., Sandri, T., Sassano, D., ... Ahluwalia, A. (2011). Substrate stiffness influences high resolution printing of living cells with an ink-jet system. *Journal of Bioscience and Bioengineering*, 112(1), 79–85.
- Tuan, R. S., Chen, A. F., & Klatt, B. A. (2013). Cartilage regeneration. *The Journal of the American Academy of Orthopaedic Surgeons*, 21(5), 303–311.
- Van Hoorick, J., Tytgat, L., Dobos, A., Ottevaere, H., Van Erps, J., Thienpont, H., ... Van Vlierberghe, S. (2019). (Photo-)crosslinkable gelatin derivatives for biofabrication applications. *Acta Biomaterialia*, 97, 46–73.
- Venn, M. F. (1978). Variation of chemical composition with age in human femoral head cartilage. *Annals of the Rheumatic Diseases*, 37(2), 168–174.
- Visscher, D. O., Bos, E. J., Peeters, M., Kuzmin, N. V., Groot, M. L., Helder, M. N., & van Zijlen, P. P. (2016). Cartilage tissue engineering: Preventing tissue scaffold contraction using a 3D-printed polymeric cage. *Tissue Engineering. Part C, Methods*, 22(6), 573–584.

- Wakabayashi, T., Matsumine, A., Nakazora, S., Hasegawa, M., Iino, T., Ota, H., ... Uchida, A. (2010). Fibulin-3 negatively regulates chondrocyte differentiation. *Biochemical and Biophysical Research Communications*, 391(1), 1116–1121.
- Waldman, S. D., Grynblas, M. D., Pilliar, R. M., & Kandel, R. A. (2003). The use of specific chondrocyte populations to modulate the properties of tissue-engineered cartilage. *Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society*, 21(1), 132–138.
- Wang, C., Yue, H., Huang, W., Lin, X., Xie, X., He, Z., ... Wang, M. (2020). Cryogenic 3D printing of heterogeneous scaffolds with gradient mechanical strengths and spatial delivery of osteogenic peptide/TGF-beta1 for osteochondral tissue regeneration. *Biofabrication*, 12(2), 025030.
- Wang, X., Partlow, B., Liu, J., Zheng, Z., Su, B., Wang, Y., & Kaplan, D. L. (2015). Injectable silk-polyethylene glycol hydrogels. *Acta Biomaterialia*, 12, 51–61.
- Weisel, J. W., & Litvinov, R. I. (2017). Fibrin formation, structure and properties. *Sub-cellular Biochemistry*, 82, 405–456.
- Wendt, D., Stroebel, S., Jakob, M., John, G. T., & Martin, I. (2006). Uniform tissues engineered by seeding and culturing cells in 3D scaffolds under perfusion at defined oxygen tensions. *Biorheology*, 43(3–4), 481–488.
- Wilson, W. C., Jr., & Boland, T. (2003). Cell and organ printing 1: Protein and cell printers. *The Anatomical Record. Part A, Discoveries in Molecular, Cellular, and Evolutionary Biology*, 272(2), 491–496.
- Wong, B. L., Kim, S. H., Antonacci, J. M., McIlwraith, C. W., & Sah, R. L. (2010). Cartilage shear dynamics during tibio-femoral articulation: Effect of acute joint injury and tribosupplementation on synovial fluid lubrication. *Osteoarthritis and Cartilage/OARS, Osteoarthritis Research Society*, 18(3), 464–471.
- Wong, M., Wuethrich, P., Egli, P., & Hunziker, E. (1996). Zone-specific cell biosynthetic activity in mature bovine articular cartilage: A new method using confocal microscopic stereology and quantitative autoradiography. *Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society*, 14(3), 424–432.
- Wyde, V., Livesey, C., & Blom, A. W. (2012). Restriction in participation in leisure activities after joint replacement: An exploratory study. *Age and Ageing*, 41(2), 246–249.
- Xu, T., Zhao, W., Zhu, J. M., Albanna, M. Z., Yoo, J. J., & Atala, A. (2013). Complex heterogeneous tissue constructs containing multiple cell types prepared by inkjet printing technology. *Biomaterials*, 34(1), 130–139.
- Yamasaki, A., Kunitomi, Y., Murata, D., Sunaga, T., Kuramoto, T., Sogawa, T., & Misumi, K. (2019). Osteochondral regeneration using constructs of mesenchymal stem cells made by bio three-dimensional printing in mini-pigs. *Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society*, 37(6), 1398–1408.
- Yang, X., Lu, Z., Wu, H., Li, W., Zheng, L., & Zhao, J. (2018). Collagen-alginate as bioink for three-dimensional (3D) cell printing based cartilage tissue engineering. *Materials Science Engineering: C Materials for Biological Applications*, 83, 195–201.
- Young, A. T., White, O. C., & Daniele, M. A. (2020). Rheological properties of coordinated physical gelation and chemical crosslinking in gelatin methacryloyl (GelMA) hydrogels. *Macromolecular Bioscience*, 20(12), e2000183.
- Zanetti, M., Ratcliffe, A., & Watt, F. M. (1985). Two subpopulations of differentiated chondrocytes identified with a monoclonal antibody to keratan sulfate. *The Journal of Cell Biology*, 101(1), 53–59.
- Zeifang, F., Oberle, D., Nierhoff, C., Richter, W., Moradi, B., & Schmitt, H. (2010). Autologous chondrocyte implantation using the original periosteum-cover technique vs matrix-associated autologous chondrocyte implantation: A randomized clinical trial. *The American Journal of Sports Medicine*, 38(5), 924–933.
- Zhang, X., Zhai, C., Fei, H., Liu, Y., Wang, Z., Luo, C., ... Fan, W. (2018). Composite silk-extracellular matrix scaffolds for enhanced chondrogenesis of mesenchymal stem cells. *Tissue Engineering. Part C, Methods*, 24(11), 645–658.

Bioprinting for Skin

15

Lothar Koch^{1,2,*}, Stefanie Michael^{3,*}, Kerstin Reimers^{3,†}, Sarah Strauß³, Peter M. Vogt³ and Boris Chichkov^{1,2}

¹*Institute of Quantum Optics, Leibniz University Hannover, Hannover, Germany*

²*Lower Saxony Centre for Biomedical Engineering, Implant Research and Development, Hannover, Germany*

³*Department of Plastic, Hand- and Reconstructive Surgery, Hannover Medical School, Hannover, Germany*

15.1 SKIN, SKIN SUBSTITUTES, POSSIBLE APPLICATIONS FOR PRINTED SKIN

15.1.1 SKIN—FUNCTION AND STRUCTURE

15.1.1.1 Function

The skin is the largest organ, covering the whole body and having a surface area of 1.5–2 m² and a weight of 3–10 kg. Due to its specific structure, it represents a barrier between the environment on the outside and the inside of the person and thereby embodies a very effective means for the protection against environmental harms such as chemical, mechanical, and thermal influences, irradiation, or pathogens.

The first protection against pathogens is achieved by the acid mantle of the skin, with a pH of 5.5–5.7. It is complemented by antimicrobial peptides (defensins) of the innate immune system and several cell types of the adaptive immune system (Langerhans cells, macrophages, leukocytes, plasma cells, and mast cells). Furthermore, the apical surface of the skin is inhabited by various bacteria and fungi, which constitute the normal skin flora and contribute to its protecting function. As commensals, they protect their habitat—and thereby us—against pathogenic microorganisms (Michael, 2013).

Apart from its protective function against external influences, the skin is crucial for the maintenance of temperature and water balance homeostasis, avoiding, for example, dehydration or hypothermia. In case of large burn injuries, the barrier function of the skin is destroyed and liquid and protein loss may result in a life-threatening condition.

Moreover, the skin acts as a sensory organ, being able to detect pain, contact, pressure, vibration, itching, and temperature. This contributes not only to our protection but also to our general perception of the environment by complementing, for example, visual or auditory perceptions.

*These authors contributed equally.

†Deceased December 23, 2015.

On the social level, the skin is essential in representation, communication, and displaying the current mood. Thereby it is important for our daily life and the contact and interaction between each other. In this context, the skin color may state a person's likings and habits, for example, having deeply tanned skin due to staying outside often alternatively to regularly visiting a solarium. Also the hair can send optical signals with social, cultural, psychological, or political content, depending on its form, color, presence, or absence (Michael, 2013).

15.1.2 STRUCTURE

The skin is composed of four layers, namely the epidermis, the basement membrane, the dermis, and the hypodermis Fig. 15.1. All of these structures, except the basement membrane, contain various specialised cell types and serve different purposes.

15.1.2.1 Epidermis

The epidermis is the uppermost skin layer and consists of a multilayered stratified squamous epithelium formed by keratinocytes. Depending on its location the epidermis is normally about 0.05 mm thick, but may reach several millimeters at the palms and the sole of the feet (Michael, 2013). Throughout life the epidermis regenerates continuously, having a turnover rate of about 30–56 days. It is composed of four layers: the basal layer, the spinous layer, the granular layer, and the cornified layer (from inside to outside). In the basal layer, epidermal stem cells are responsible for

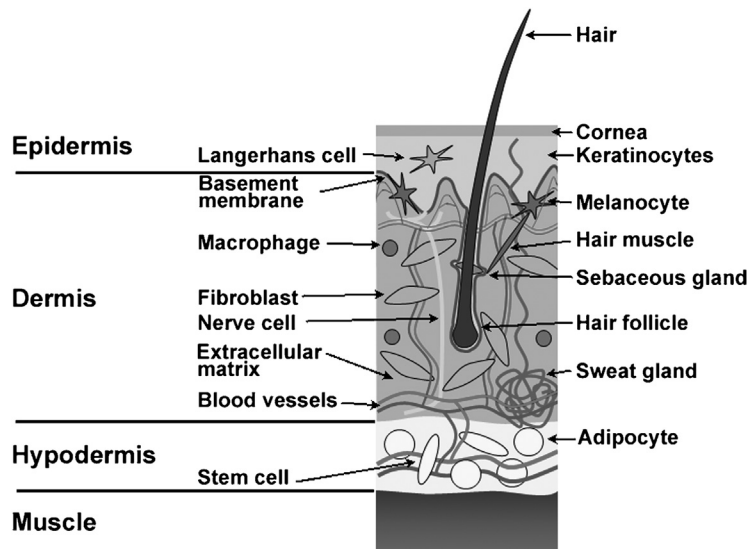


FIGURE 15.1

The image shows the skin with its specific and complex 3D structure. It consists of several layers: the epidermis, which is tightly connected to the dermis via the basement membrane, the dermis and the hypodermis. Specific cell types and organs are present in the different layers, exhibiting specific functions.

the regenerative capacity of the skin. These cells are highly proliferative, characterized by cytokeratin 5 and 14 expression, and comprise interfollicular stem cells, hair follicle stem cells, and sebaceous gland stem cells (Koster, 2009). They are the source of all keratinocytes which form the epidermis.

During their life span, keratinocytes undergo terminal differentiation, which is accompanied by many biochemical and morphological changes (Houben, De Paepe, & Rogiers, 2007; Koster, 2009; Proksch, Brandner, & Jensen, 2008). After a few more replications, the cells lose their replicative capability and start to produce different proteins, for example, cytokeratins 1 and 10. Filaggrin helps the cytokeratins to form tight bundles of intermediate filaments that are important for the structural integrity of the cell and finally promote the collapse of the cell into a flattened shape (Houben et al., 2007). A so-called cornified envelope develops, including proteins such as involucrin, loricrin, and filaggrin as well as different lipids (Houben et al., 2007). At that stage, both, proteins and lipids, are covalently linked (Eckert, Sturniolo, Broome, Ruse, & Rorke, 2005). During this transition to so-called corneocytes, the cells undergo programmed cell death and are finally embedded in a self-produced intercellular lipid matrix, comprising cholesterol, free fatty acids, and other lipids. These tightly packed lipids are responsible for the low permeability of skin to water and the formation of a permeability barrier. Nevertheless, the lower layers also contribute to the barrier function via the formation of tight junction. Several cells together form horny flakes, the uppermost layer of the skin, which are shed continuously in humans (Houben et al., 2007).

If the balance between proliferation and differentiation is impaired, a disturbance of the skin barrier may occur, resulting in the formation of inflammation, dermatitis, ichthyosis, psoriasis, and the entry of pathogens (Proksch et al., 2008). Therefore, in healthy persons, it is controlled tightly.

To form a stable epithelium, the cells are connected via cell–cell and cell–matrix junctions. Adherens junctions and desmosomes provide a mechanical connection and thereby mechanical strength between neighboring keratinocytes, while hemidesmosomes and actin-linked cell–matrix adhesions are responsible for the coupling of cells to the underlying basement membrane. In adherens junctions, so-called classical cadherins are found, especially e-cadherin in skin. The connection to the basement membrane is realized by integrins. Tight junctions also contribute to the barrier function of the skin by closing the intercellular space between the apical and the basal part of the epithelium. The barrier, however, has a selective permeability, which can be altered depending on the circumstances. Moreover, gap junctions form channels between the cytoplasm of neighboring cells via transmembrane proteins (connexins). They allow for the exchange of low-molecular substances (e.g., hormones, glucose, or vitamins) as well as ions (e.g., electrical coupling in the heart muscle) and furthermore are essential for cell–cell communication.

Other cell types in the epidermis are, for example, melanocytes, which reside in the basal layer. Due to their production of melanin and its distribution to the surrounding keratinocytes, they are responsible for the skin color as well as protection against ultraviolet (UV) irradiation and corresponding mutagenesis. Furthermore, Langerhans cells can be found in the epidermis. They belong to the adaptive immune system and represent the antigen presenting cells of the epidermis (Michael, 2013).

Since the epidermis does not contain any blood vessels, it needs to be supplied with nutrients and oxygen via diffusion from the dermis. At the dermal–epidermal junction, both layers

interdigitate, forming the rete ridges (epidermal part) and the papillae (dermal part). The resulting high increase of the available surface for diffusion enables the adequate supply of the epidermis. Furthermore, the rete ridges/papillae are essential for the mechanical stability of the skin because they greatly increase the resistance of skin against shear forces (Michael, 2013).

15.1.2.2 Basement Membrane

The basement membrane is essential for the attachment of the epidermis to the dermis and thereby the mechanical strength of skin. Moreover, it stabilizes the connection between the epithelial cells of the basal layer and prevents them from sliding apart. Furthermore, it promotes cell survival, proliferation, and differentiation of the cells. The basement membrane consists of special extracellular matrix (ECM) molecules and is partly secreted by the epidermal cells and partly by the dermal cells. It contains, for example, collagen types IV and VII, glycoproteins such as laminin and fibronectin, nidogen, proteoglycans, and integrins. Collagen type IV forms the basis of the membrane, being formed like a felt-like network. It gives the basement membrane and its high tensile strength. The other components are mostly integrated in this network. To attach the membrane to the underlying dermis, collagen type VII forms anchoring fibrils (Michael, 2013).

The basement membrane acts as a barrier between the epidermis and the dermis, keeping the keratinocytes and fibroblasts in their spatial surrounding. In contrast, macrophages, lymphocytes, and nerve cells can cross the membrane by secreting specialized enzymes, which are able to degrade components of the membrane. Of course, nutrients, oxygen, and metabolites must be able to pass the barrier (Michael, 2013).

15.1.2.3 Dermis

Underneath the basement membrane, the dermis is located. Its thickness varies between 0.6 mm at the eyelids and more than 3 mm at the back and the sole of the feet. The dermis is divided into two parts with the upper papillary region containing most of the cells and the lower reticular region comprising hair follicles and glands.

The main characteristic of the dermis is the abundant presence of ECM. It is secreted by the resident cells, the fibroblasts. The dermis has several different tasks. Via the proteoglycans and glycoproteins of the ECM, liquid in form of hydrated water is bound. This results in a gel-like substance, which can resist compressive forces well, thereby protecting the underlying tissue. Diffusion of nutrients, metabolites, and hormones is possible, though, and serves the supply of the skin. The tensile strength and mechanical stability of the dermis and thereby the skin are based on collagen type I, which form long fibers. Of course, elasticity is also needed and effected by elastic fibers. Altogether, the dermis can absorb mechanical compression or shear forces, while simultaneously being very pliable (Michael, 2013).

In contrast to the epidermis, the dermis contains many blood vessels. They are organized in two plexuses, one being located directly above the hypodermis and the other one directly underneath the basement membrane. The latter is responsible for the supply of the epidermis via diffusion and reaches into the papillae. The blood vessel system of the dermis also takes part in temperature regulation of the whole body.

Furthermore, the dermis contains sebaceous and perspiratory glands, hair follicles, and nerves. The nerves are responsible for the perception of pain, touch, itch, and temperature. Finally, cells of the innate and the adapted immune system can also be found in the dermis: mast cells, macrophages, leukocytes, and plasma cells (Michael, 2013).

15.1.2.4 Hypodermis

The hypodermis consists of connective tissue and fat, and has several functions. First of all, it serves as heat insulation, energy store, and protection of internal organs against external mechanical forces. Furthermore, the hypodermis connects the upper skin layers to the lower tissues, for example, muscles, fascia, and bone. Moreover, hormones are generated in there. The blood vessels in the hypodermis are even larger than those in the dermis and supply the glands and hair bulges with nutrients and oxygen (Michael, 2013). As cell types, mainly adipocytes are present but also multipotent stem cells (Zuk et al., 2001) can be found.

15.1.2.5 Cutaneous Appendages

As already mentioned before, several cutaneous appendages can be found in the skin, which are formed by specific cells: hair bulges/hair, nails, sebaceous glands, perspiratory glands, scent glands, and mammary glands. Mammary glands serve the feeding of the offspring, of course. Perspiratory glands produce sweat and thus are crucial for temperature homeostasis and regulation. Specialized types of hair can be found in different locations of the body, being hormone dependent and comprising eyelashes, eyebrows, beard hairs, pubic and axillary hair, and hair in the nose or the outer ear canal. Sebum secreted by the sebaceous glands keeps the skin and the hair pliable. Apart from playing a role in temperature control, hair is also involved in tactile sensation. Nails are a further specialized form of hair. They protect us against mechanical injuries and also support the tactile ability of skin (Michael, 2013).

15.2 SKIN SUBSTITUTES, APPLICATIONS FOR PRINTED SKIN

Skin substitutes/equivalents may have several applications. These comprise wound healing after skin injuries as well as research of skin diseases or corresponding drugs.

15.2.1 INJURIES OF THE SKIN

If the skin is harmed superficially, it will normally heal by itself without complications. But under certain circumstances, even the skin cannot regenerate any more. This is true for large and/or deep burn injuries. Burns occur in many different situations of life and reach from simple sunburns to scalding with hot liquids and electrical or chemical accidents. Also, direct heat, irradiation or friction can cause burns (Herndon, 2002; Pallua & von Bülow, 2006; Chapter 2). Unfortunately, treatment of large and deep burns is only of limited success. Usually, autologous split-thickness skin grafts are used to cover the wounds, but donor sites are rare in large burns and extensive scarring can often not be avoided. This may lead to esthetic and functional impairment. In case of deep wounds, dermal substitutes are used but insufficient vascularization may lead to rejection of the graft. Moreover, available skin substitutes do not contain hair follicles, melanocytes, and sebaceous and sweat glands, which results in a lack of the according functions such as temperature regulation and esthetic appeal. Altogether, this may result in a low quality of life for the patients including fear of defacement, rejection, and failure and feelings of worthlessness. In severe cases, scar formation may also lead to impairment of mobility and therefore restrictions in everyday life or at work.

Another problematic situation results from chronic wounds that are characterized by their pathophysiology. The normally tightly controlled wound healing is impaired, which results in badly or nonhealing wounds. Chronic wounds comprise three different categories: an impaired venous drain due to a faulty microcirculation (venous ulcers of the lower extremities), an increased mechanical strain (decubitus), and a vascular, nerval, and/or metabolic tissue injury (diabetes). Often, chronic wounds are the result of several different causes (Riedel, Ryssel, Koellensperger, Germann, & Kremer, 2008). Pain and exudates may cause constrictions of mobility, leading to restrictions at work or in everyday life. Similar fears and feelings as accompany burn patients may occur. Nonchronic but deep and large wounds can also result from tumor resections or accidents.

In all described cases, a skin substitute as similar to physiological skin as possible is needed to be able to replace the lost skin adequately.

15.2.2 RESEARCH

Also, in research, skin equivalents are used for different purposes. First of all, they can be applied to replace animal experiments in the development of cosmetics, cleaning agents, and drugs. In the European Union, animal experiments for testing of cosmetics are illicit. Therefore another reliable test system is needed to assess the newly developed components. Observed parameters are inflammations, irritancies, or toxic effects (Gibbs, 2009; Mertsching, Weimer, Kersen, & Brunner, 2008). The disadvantage of skin equivalents in this context is that no complete organism is present and thus no systemic reaction can be judged. On the other hand, human cells can be used for *in vitro* test systems, thereby avoiding wrong results due to species specificities (Kuhn, Radtke, Allmeling, Vogt, & Reimers, 2011).

Furthermore, skin equivalents can be used as a model for (skin) diseases, giving insights into the mechanisms of the disease and/or testing corresponding drugs. In this context, they can comprise different kinds of cells and materials. For example, photo carcinogenesis due to UV irradiation can be assessed in skin equivalents including cells from healthy persons compared to cells from *xeroderma pigmentosum* patients. These suffer from an impaired DNA repair system and are especially prone to skin cancer (Bernerd et al., 2001). Invasion and metastatic potential of skin cancer types may be assessed by including these cell types in the skin equivalents. In this context, the three-dimensional (3D) structure including the basement membrane is crucial since cancer cells from the epidermis need to be able to pass the basement membrane to develop their invasive potential (Kataoka, Umeda, Shigeta, Takahashi, & Komori, 2010). Less dangerous, but also very unpleasant are pigmentation disorders, which can also be studied using skin equivalents (Okazaki, Suzuki, Yoshimura, & Harii, 2005). Furthermore, skin equivalents can be used as an *in vitro* model of psoriasis (Fransson, 2000), to assess antifungal drugs for the treatment of candidiasis (Okeke, Tsuboi, & Ogawa, 2001), or to test new treatments for wound healing (Xie et al., 2010).

15.3 SKIN SUBSTITUTES GENERATED BY BIOPRINTING

Very important in any case is the organotypic 3D structure of the skin equivalent, mimicking natural skin and giving the possibility for as naturally an interaction of the different skin cell types as possible. This specific spatial distribution of the several cell types can be achieved with bioprinting.

For printing skin cells, all three most common bioprinting techniques such as microdispensing (extrusion printing), inkjet printing, and laser-assisted bioprinting have been applied. For inkjet bioprinting, mostly commercial office inkjet printers are modified for cell printing. However, inkjet systems for liquids other than ink are now increasingly being offered commercially, some of which have also been used to print living cells. Microdispensing uses extrusion mechanisms (Khalil, Nam, & Sun, 2005; Lee et al., 2009). The material is extruded under pressure out of a nozzle with about 50–1000 μm diameter continuously or in droplets by switching a microvalve with a suitable frequency. Laser-assisted bioprinting is explained below (Section 15.3.3).

15.3.1 HYDROGEL

For printing living cells with one of these techniques, the cells are embedded in a medium, mostly a hydrogel or a hydrogel precursor, also called sol, such as collagen, fibrinogen, hyaluronic acid, alginate, or similar. They are printed as a hydrogel droplet containing between one and several hundred cells or extruded as a hydrogel strand with embedded cells.

If 3D cell patterns or tissues are to be printed, the hydrogel (precursor) has to fulfill several requirements. First, it needs to be suitable for the printing process. This includes an appropriate viscosity, which depends on the concentration and material-specific properties. Shear-thinning gels or gel precursors are advantageous to reduce the shear forces during the printing process, while shear-thickening gels are problematic for all discussed printing technologies, since the shear forces increase disproportionately high with the velocity of the gel, which is accelerated during the printing process. To avoid cell damage, lower gel viscosities are required. Alginate and hyaluronic acid are shear-thinning gels, which are often used for cell printing. Also, the wetting characteristics of the gel (precursor) on the printing setup's surface materials are important. Second, the gel should support cell survival by a convenient environment. Therefore a neutral pH value is mandatory. Third, after printing, the gel functions as the extracellular matrix, which in 3D patterns requires a certain stiffness and mechanical strength. Fourth, the gel must not affect the cells undesirably, since in general cells respond to environmental cues. There can be manifold stimuli having physical, mechanical, chemical, and biological backgrounds.

In general, to fulfill these requirements, the hydrogel consists of four components. A primary component, offering a suitable environment with nutrients for the cells, is mixed with a secondary component for optimizing the viscosity. Depending on the application, as a third component stimuli like growth factors or agents are added. In this mixture, called hydrogel precursor or sol, the cells are suspended. This hydrogel precursor with the embedded cells is then printed in the predefined pattern. Afterwards, a crosslinker is printed for gelation to attain the desired stiffness as the extracellular matrix.

Examples are (1) fibrinogen mixed with hyaluronic acid (to have a suitable viscosity for printing) and cross-linked with thrombin or (2) blood plasma mixed with alginate and cross-linked with calcium chloride. By this means, hydrogels and hydrogel precursors with a wide range of rheological properties (Grüne, Unger, Koch, Deiwick, & Chichkov, 2011; Lin, Huang, & Chrisey, 2009) have been printed. The crosslinker can be printed or sprayed onto the printed hydrogel precursor in a second step, alternatively the sol can be printed into a crosslinker reservoir (Yan, Huang, & Chrisey, 2013).

However, some gels of interest like collagen, the most abundant protein in mammals, do not allow a postprint gelling. Collagen is gelled by a change of the pH value. Therefore a complete merging of all gel components before printing is required, since the cells will not survive in the acidic collagen precursor. Thus the ability of printing high-viscosity gels is needed.

However, the printing technologies have different viscosity limitations for printing living cells, since shear forces, especially in printing nozzles, may destroy or affect the cells. Thus inkjet printing is limited to low viscosities (<0.1 Pa s) and low cell densities (up to a few million cells per milliliter) to avoid shear stress in the nozzle and cell clogging (Born, Zhang, Al-Rubeai, & Thomas, 1992). For microdispensing of cells, nozzles with about 50–1000 μm diameter have been applied. With smaller nozzle diameters similar limitations in viscosity and cell density as with inkjet printers exist, but these limits can be varied by the extrusion velocity as a further parameter. With larger extrusion nozzle diameters, higher viscosity and cell density can be printed at the expense of resolution (Chang, Nam, & Sun, 2008).

15.3.2 EXTRUSION-BASED BIOPRINTED SKIN CELLS

Lee et al. (2009) successfully printed vital human skin fibroblasts and keratinocytes with an extrusion printer. Therefore they printed 10 layers of collagen precursor (rat tail collagen type I, BD Biosciences, Billerica, MA, United States). Since this precursor is acidic, they printed it without cells. Each layer was cross-linked with nebulized aqueous sodium bicarbonate (NaHCO_3). In between these layers, they printed one layer of fibroblasts and (after six further collagen layers) one layer of keratinocytes, each suspended in cell culture medium. Thus they circumvented the general problem with collagen printing. Collagen is the most abundant protein in the human skin (and in the human body) and therefore, it is of major interest in tissue engineering. However, the collagen precursor is acidic and after neutralization with a base, which is indispensable for cell survival, the viscosity of the collagen gel increases, thus that it cannot be printed with small nozzles anymore.

On the other hand, by printing the cells, suspended in cell medium, separately from printing the collagen, it might be difficult to achieve a tissue-like (especially epithelium-like) cell density since the intermittent collagen layers serve as a scaffold but do not contain cells. Besides, the high volume of the cell media layers might cause an ease of displaceability between the collagen layers and thus avoid the formation of a stiff 3D hydrogel block.

A peculiarity of the work of Lee et al. is the printing in a 3D free-form mold of poly(dimethylsiloxane). The intention is to replicate the surface contour of a skin wound with the mold. The single layers of fibroblasts and keratinocytes were meant for the demonstration of the “ability to print spatially distinctive cell layers.” However, they did not show tissue generation or the establishment of intercellular junctions. Furthermore, the gap of about 75 μm between fibroblasts and keratinocytes does not allow the development of an interface (basement membrane) because it can be found between dermis and epidermis in natural skin.

Cubo, Garcia, del Cañizo, Velasco, and Jorcano (2016) used extrusion-based bioprinting of human plasma as well as primary human fibroblasts and keratinocytes obtained from skin biopsies, to print a human bilayered skin; this combination of human plasma and primary cells is already used for the clinical treatment of burn wounds.

Liu et al. (2016) printed 3D porous structures with defined pore structure and integrated epithelial progenitor cells and inductive factors for sweat gland reconstruction, however, without printing of further skin cells. They observed differentiation toward sweat gland cells and pore size dependence of sweat gland morphogenesis. This study demonstrated that 3D-printed architecture effects cell behavior in glandular tissue regeneration.

A study involving many different skin cells was recently presented by Jorgensen et al. (2020). They used an extrusion printer with 500 μm diameter nozzle and printed human keratinocytes, melanocytes, fibroblasts, dermal microvascular endothelial cells, follicle dermal papilla cells, and adipocytes, suspended in fibrinogen bioink combined with gelatin, glycerol, and hyaluronic acid, to form a tri-layered skin structure (hypodermis, dermis, and epidermis). After 4 days of *in vitro* culture, they implanted these skin structures in full thickness skin wounds of athymic nude mice. They observed the formation of epidermal barrier and dermis. Untreated wounds or wounds treated with hydrogel only, for reference, showed only sparse epidermal covering, a thin epidermal layer, and low cellularity. This study also includes investigation of collagen ECM architecture generated by the printed cells; a dermal ECM consisting of mature and immature collagen fibers in a “basket-weave” orientation similar to the normal skin. In contrast, in untreated and hydrogel only treated wounds, thick parallel fibers were observed, characteristic for hypertrophic scars.

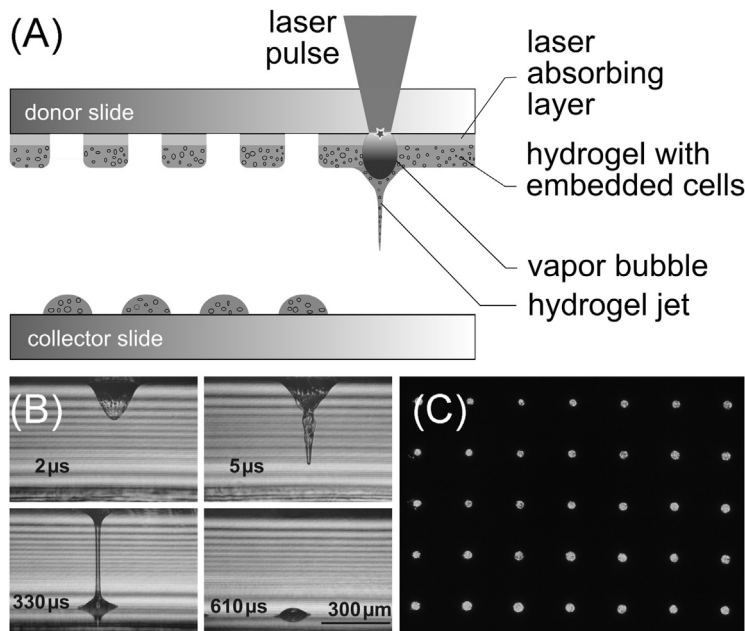
15.3.3 LASER-ASSISTED BIOPRINTED SKIN

15.3.3.1 Schematic of the Laser-Assisted Bioprinting Setup

A further technique used by several groups is laser-assisted bioprinting. In principle, a laser-bioprinting setup consists of a pulsed laser and two coplanar glass slides. The upper one (here called donor) is coated with a thin layer of a laser absorbing material, and subsequently a layer of the biomaterial that will be printed (i.e., the cells embedded in an appropriate hydrogel). Then, this glass slide is mounted upside-down above the second glass slide (here called collector). The distance is set from a few hundred microns up to a few millimeters.

As absorption layers, gold, titanium, polymers like triazene (Schiele et al., 2010), or gelatin (Schiele, Chrisey, & Corr, 2011) have been used; also systems with two layers (Lin, Huang, & Chrisey, 2011) or without absorbing layer (Barron, Ringeisen, Kim, Spargo, & Chrisey, 2004) have been described. In the latter case, the cell-containing hydrogel itself is used as the laser absorbing material. A detailed description of the different realizations and denominations of laser-bioprinting are given by Schiele et al. (2010) (Fig. 15.2).

The laser pulses are focused through the donor glass slide into the absorbing layer, which is evaporated locally in the focal spot. The high pressure vapor expands as a bubble into the gel layer and propels the subjacent gel toward the collector glass slide. This bubble reaches its maximum size and recollapses after a few microseconds, due to the vapor pressure decreasing below the outer pressure. However, the gel underneath the bubble moves forward further on by inertia. Thereby a gel jet forms, which lasts for a few hundred microseconds. Via this jet between a few picoliters and a few nanoliters of gel (with embedded cells) is transferred to the collector glass slide and remains there as a droplet (Duocastella, Fernández-Pradas, Morenza, & Serra, 2009; Unger, Gruene, Koch, Koch, & Chichkov, 2011). By moving the glass slides and the laser beam, any desired two-dimensional pattern and layer-by-layer also any 3D pattern can be generated. Fig. 15.2 shows a

**FIGURE 15.2**

Laser-assisted bioprinting: Image (A) presents a schematic sketch of the laser-assisted bioprinting setup. The laser pulse is focused through the donor glass slide into the absorbing layer, which is evaporated immediately. Thereby, a vapor bubble is generated, as can be seen in image (B). The subjacent hydrogel is accelerated by the expanding bubble. Although the bubble recollapses within about 6 μs , the hydrogel moves on due to inertia and flows within a few hundred microseconds as a “jet” onto the donor slide, where it remains as a droplet (Unger et al., 2011). The droplets can be precisely positioned as can be seen in image (C). The diameter of the droplets, each containing several cells, is about 80 μm , the distance between two droplets is 600 μm . The horizontal lines in image (B) are interference patterns resulting from the illumination of the process with coherent laser light.

printed pattern of droplets with several fibroblast cells. Fibroblasts labeled with green fluorescent protein and keratinocytes labeled in red with mCherry were embedded in a sol mixture of fibrinogen and hyaluronic acid and printed onto a layer of fibrin with a spacing of 600 μm between droplets with the same cells.

Often, the collector glass slide is coated with a hydrogel layer before printing to achieve a humid environment for the cells and to cushion the impact. However, the cells survive the printing process also without this hydrogel layer. Instead of the collector glass slide, other items can be put under the upper glass slide to print onto or into, for example, a scaffold (Ovsianikov et al., 2010).

The printed droplet volume can be controlled by the thickness and viscosity of the biomaterial layer on the donor glass slide, the thickness of the absorption layer, the laser pulse energy (Gruene, Unger, et al., 2011; Guillotin et al., 2010), and the focal spot size. The ratio between

printed droplet volume and laser pulse energy is nearly proportional in the relevant energy range at a constant viscosity and layer thickness. In contrast, there is no simple dependency of the printed droplet volume on viscosity or layer thickness at unvaried laser pulse energies. A specific viscosity for every layer thickness exists, at which the printed droplet volume reaches its maximum. This specific viscosity increases with hydrogel layer thickness. Usually, the quantity of cells per droplet is proportional to the droplet volume and the initial cell density in the hydrogel layer on the donor slide and is subject to statistical variations. The alternative of printing single cell droplets requires a low cell density and is time consuming, since each cell needs to be targeted separately. The number of printed droplets per second depends on the laser pulse repetition rate and the velocity of the mechanical setup and might be in the kilohertz range. For printing of tissue as well as high-throughput assembly of cell arrays to study multiple cell responses, in parallel, high printing speeds are needed.

Koch et al. (2010) used a laser-assisted bioprinting technique for the printing of skin. For the printing process, a Nd:YAG-laser (DIVA II; Thales Laser, Orsay, France) with 1064 nm wavelength, about 10 ns pulse duration, and 20 Hz repetition rate was used. The laser pulses were focused with a 60 mm achromatic lens into an ablation spot size with 45 μm diameter full width at half maximum (FWHM). The laser pulse energy was set to 40 μJ , corresponding to laser fluency of averaged 1.26 J/cm² in the focal spot. As an absorption layer, a 60 nm thin gold layer was deposited on the donor glass slide by sputter coating and a 60 μm thick layer of collagen with embedded skin cells was coated onto the absorption layer by blade coating.

15.3.3.2 The Printing Process Does not Affect the Cells

Several groups demonstrated successful cell printing with various cell types. However, for applications, it is essential that the printing process does not affect the cells in their vitality, behavior, genotype, and phenotype. Therefore the impact of the printing process on skin cells and other cell types was extensively studied. For the skin tissue printing described below, NIH3T3 fibroblasts and HaCaT keratinocytes were used. Hence, investigations on the effect of the printing process on these cell lines are discussed here.

The cell survival rate was examined directly after printing (Koch et al., 2010). Thereby, a survival rate of 98.4% $\pm$ 0.8% for NIH3T3 and 98.6% $\pm$ 0.3% for HaCaT has been calculated, which is in accordance with studies on other cell types (Hopp et al., 2005). Furthermore, genotoxicity was investigated via a single cell gel electrophoresis (Comet Assay). It was demonstrated that the printing does not induce DNA strand breaks (Koch et al., 2010). The genotypes of the cells remained unaffected. These findings are consistent with similar investigations by Ringeisen et al. (2004) with P19 pluripotent embryonal carcinoma cells. Apoptosis as a parameter of possible cell death caused by LaBP was assessed by measurement of the activity of caspases 3/7. Up to 48 hours after printing, no increase in apoptosis was detected, neither compared to nonprinted control cells nor compared at different test intervals (Koch et al., 2010). Also, the influence of the printing process on cell proliferation was studied by cell counting up to 6 days after printing. In accordance with experiments of other groups on other cell types (Barron, Krizman, & Ringeisen, 2005; Hopp et al., 2005), no difference in the proliferation behavior of NIH3T3 fibroblasts and HaCaT keratinocytes compared to nonprinted control cells could be found (Koch et al., 2010). Since high temperature is induced in the absorption layer by the laser pulse energy, potential cell damage by heat was investigated via immunocytochemical studies (not shown). No increased expression of heat shock proteins

by printed cells was demonstrated (Gruene, Deiwick, et al., 2011). This is in line with experiments of other groups (Barron et al., 2004, 2005). Furthermore, in studies with stem cells, it was shown that the printing process does not affect the immunophenotype (Koch et al., 2010) or the differentiation potential (Gruene, Deiwick, et al., 2011; Gruene, Pflaum, et al., 2011) of the printed cells. Summarized, all studies so far consistently show no cell affection by the laser printing procedure, printed cells are viable and fully functional.

15.3.3.3 Laser-Assisted Printing of Skin Tissue—In Vitro Culture

For printing 3D skin tissue, mouse NIH3T3 Swiss albino fibroblast (DSMZ Braunschweig, Germany) and human immortalized HaCaT (DKFZ, Heidelberg, Germany) keratinocyte cell lines were used (Koch et al., 2012; Michael, Sorg, Peck, Koch, et al., 2013). These well-established cell lines have been combined in other studies, too (Bigelow, Jen, Delehedde, Chari, & McDonnell, 2005; Delehedde et al., 2001). 3T3 fibroblast cells are often used in keratinocyte cultivation because of secreting growth factors favorable for keratinocytes (Boehnke et al., 2007; Linge, 2004; Schoop, Mirancea, & Fusenig, 1999).

To approximate native skin as far as possible, collagen type I from rat tail was applied as hydrogel for embedding the cells for the printing process and as extracellular matrix afterwards, since this is the main extracellular matrix protein in skin (Koch et al., 2012).

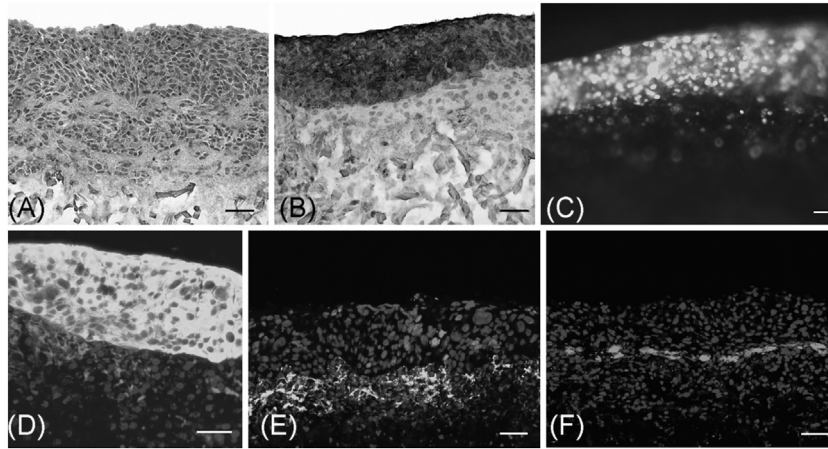
As the basis for the printed skin tissue, a 1 mm thick sheet of a collagen–elastin matrix (Matriderm, MedSkin Solutions Dr. Suwelack AG Billerbeck, Germany) was used for providing mechanical strength directly after printing. Matriderm is porous and permeable for cell culture media (Golinski et al., 2009). Successively, 20 layers of NIH3T3 fibroblasts and 20 layers of HaCaT keratinocytes, both embedded in collagen, were printed onto the Matriderm substrate. After printing, the skin constructs were cultivated for 10 days submerged in cell media. Histologic sections, depicted in Fig. 15.3, showed a tissue-like cell pattern. The printed cells remained in the bilayered 3D structure. Furthermore, Fig. 15.3 also depicts the formation of a basal lamina as part of a basement membrane at the interface between keratinocytes and fibroblasts, as it exists between epidermis and dermis in natural skin. Basal laminae are established by epithelial cells like keratinocytes and mainly consist of the proteins collagen type IV and laminin, which are connected to the epithelial cell membranes.

Directly after printing, the 40 printed layers had a total thickness (without the Matriderm) of about 500 μm . This thickness decreased to 250 μm or 50% within 24 hours due to the fibroblasts contracting the surrounding collagen. This shrinkage is well known from literature (Bellows, Melcher, & Aubin, 1982). The remaining thickness of 250 μm is small enough to supply the cells by diffusion.

15.3.3.3.1 Tissue Formation *In Vitro* (Submerged Culture)

Tissue formation is determined by intercellular junctions (Ko, Arora, Lee, & McCulloch, 2000), which can be found as cell–cell and cell–matrix connections in all kinds of tissue, abundantly in epithelium like the epidermis.

Adherens junctions occur in epithelium often as bands that encircle the cell (zonula adherens), while in other tissues normally only punctuate junctions, spots of adhesion, can be seen. Adherens junctions are composed mainly of cadherins (calcium-dependent adherent proteins), which are connected to the actin cytoskeleton of the cells via other proteins. At the extracellular side of the cell

**FIGURE 15.3**

Laser-printed skin constructs: skin mimicking bi-layered construct: 20 layers of murine fibroblasts (NIH3T3) and 20 layers of human keratinocytes (HaCaT) embedded in collagen type I were printed subsequently on a sheet of a Matrigel collagen–elastin matrix. A section through the laser-printed structure, prepared directly after printing, with transduced fibroblasts (red) and keratinocytes (green), is depicted as image (C). The images (A), (B), (D), (E), and (F) show cryostat sections, prepared 10 days after printing. All scale bars are 50 μm . A haematoxylin and eosin staining (A) shows all printed cells in a tissue-like pattern.

Immunoperoxidase staining of cytokeratin 14 in reddish-brown (B) depicts keratinocytes in the bi-layered structure while all cell nuclei (fibroblasts and keratinocytes) are counterstained in light blue with haematoxylin. In picture (D) the fibroblasts are stained in red (pan-reticular fibroblast), keratinocytes are stained in green (cytokeratin 14), and cell nuclei are stained in blue (Hoechst 33342). Especially the keratinocytes formed a compact cell organization. In picture (E), all cell nuclei are stained with Hoechst 33342 (blue), the proliferating cell nuclei are stained with Ki-67 (red), and fibroblasts are stained in green. The fibroblasts and the keratinocytes above are still vital and proliferating. Image (F) depicts an antilaminin staining in green and all cell nuclei in blue (Hoechst 33342). Laminin is a major constituent of the basement membrane in skin.

Reprint from Koch et al. (2012). Copyright © 2012 Wiley Periodicals, Inc.

membrane, cadherins connect with cadherins of adjacent cells by forming homodimers. Adherens junctions are essential for tissue morphogenesis and cohesion (Gumbiner, 1996; Niessen, 2007).

Gap junctions are clusters of cell–cell channels crossing the cell membranes of two adjacent cells and connecting their cytoplasm directly. They enable intercellular communication between neighboring cells by exchanging small molecules (≤ 1000 Da) by diffusion and synchronising electrical as well as physiological activities (Mese, Richard, & White, 2007; Simon & Goodenough, 1998). Gap junctions are established by two cells, each forming one semichannel (connexon). Connexons consist of normally six connexins (Richard, 2000) in a hexagonal pattern with a free channel in the middle. The connexons of two adjacent cells connect forming one intercellular channel. Gap junctions play a fundamental role in differentiation, cell cycle progression, and cell survival (Fitzgerald et al., 1994; Schlie, Mazur, Bintig, & Ngezahayo, 2010).

Therefore cadherin and connexin localization as well as gap junction coupling are good parameters to study tissue properties. Thus tissue formation of laser-printed 3D skin structures was studied with a focus on cadherins and connexin-43 (Cx43), which is the main connexin in human skin (Fitzgerald et al., 1994; Wiszniewski, Limat, Saurat, Meda, & Salomon, 2000). The functionality of cell–cell communication via gap junction coupling was assessed with a dye-transfer, a so-called scrape-loading method (Begandt, Bintig, Oberheide, Schlie, & Ngezahayo, 2010).

The extensive formation of intercellular adherens junctions between printed keratinocytes can be seen in Fig. 15.4 by pan-cadherin staining (Koch et al., 2012). Keratinocytes form the dermal epithelium (epidermis), where a higher level of junctions can be found encircling the whole cell (Niessen, 2007). Between fibroblasts, there is a minor formation of adherens junctions (not shown).

Immunostaining (Fig. 15.4) of Cx43, the main connexin in human skin, shows its expression in all cells and its localization within the cell membrane 10 days after the cell printing procedure (Koch et al., 2012). As can be seen, Cx43 is distributed in a scattered, punctate manner, which is a sign for the formation of gap junctions (Morritt et al., 2007).

The gap junctions' functional capability for cell–cell communication was tested by a dye transfer with a so-called scrape loading method in vital 3D cell constructs. For this purpose, confluent

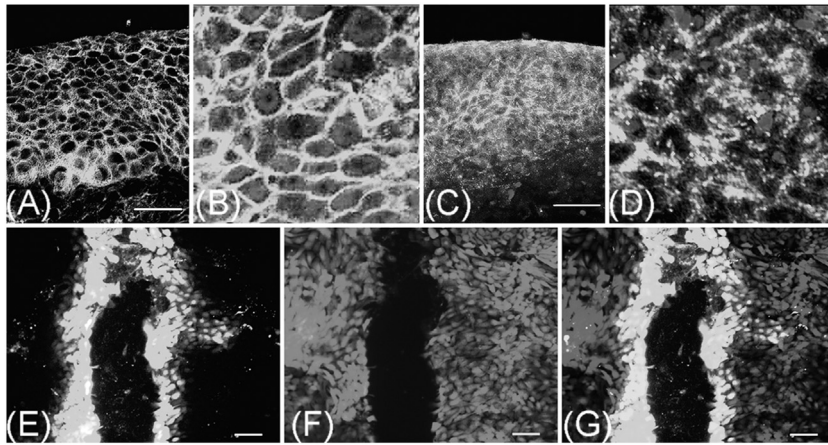


FIGURE 15.4

Laser-printed skin constructs: 10 days after printing, histological sections were prepared to investigate the formation of intercellular junctions, which are essential for tissue function. Fluorescence microscopic images of 3D laser-printed fibroblasts and keratinocytes are depicted as a qualitative analysis of adherens and gap junction formation. (A) Pan-cadherin-staining shows the adherens junctions consisting of cadherins, which are located between the membranes of neighboring cells; (B) pancadherin-staining (green) and cell nuclei staining with Hoechst 33342 (blue); (C, D) connexin-43 (Cx43) staining (green) and Hoechst 33342 nuclei staining (blue), connexins are the constituents of the gap junctions; picture (D) shows Cx43 distributed in a scattered, punctate fashion, which is a sign for the formation of gap junctions; (E–G) gap junction coupling visualized in green with Lucifer yellow (E, G) after scrape-loading procedure, the keratinocytes, transfected with mCherry, are depicted in red (F, G). All scale bars are 50 μm .

Reprint from Koch et al. (2012). Copyright © 2012 Wiley Periodicals, Inc.

grown 3D-printed keratinocyte cells were scratched with a razor blade and the dye Lucifer yellow (LY) was added, which then could penetrate into the destroyed cells. Since LY molecules are small enough to pass the gap junction channels, the penetration into channel-connected neighboring cells and the diffusion distance through further cells can be analyzed as a measure for gap junction coupling. Twenty layers of keratinocytes embedded in collagen were printed on a glass slide and cultivated for 10 days at 37°C (5% CO₂). After washing with PBS, the slides were submerged in a DPBS solution including 0.25% LY. With a razor blade, one scratch along the whole sample was set. After 5 minutes of incubation, the slides were washed four times with DPBS for 5 minutes each. The diffusion distance of LY was documented via fluorescence microscopy (Fig. 15.4). The LY dye penetrated from the scratched keratinocyte cells into neighboring cells and further cells, which is only possible through functional gap junctions. The LY went through up to 10 cells. This demonstrates that the printed skin grafts possess epithelium-specific functions with respect to adherens and gap junctions (Koch et al., 2012).

15.3.3.3.2 Tissue Formation *In Vitro* (Air–Liquid Interface Culture)

Up to now, the production of skin equivalents via laser-assisted bioprinting and their culture under submerged conditions has been described. As a first step, this served to assess the printed skin constructs and by that also the printing process. As a next step, keratinocyte differentiation, which is crucial for the formation of a multikeratinized epidermis, was induced by different stimuli. First, the skin constructs were raised to the air–liquid interface (Michael, Sorg, Peck, Koch, et al., 2013). This means that the skin equivalents were supplied with nutrients and liquid from below but they had contact to the air from above—like real skin in our bodies. Second, differentiation supporting substances were added (10⁻⁷ mM hydrocortisone, 10⁻⁷ mM isoprenaline hydrochloride, and 10⁻⁷ mM insulin) and the calcium concentration was raised.

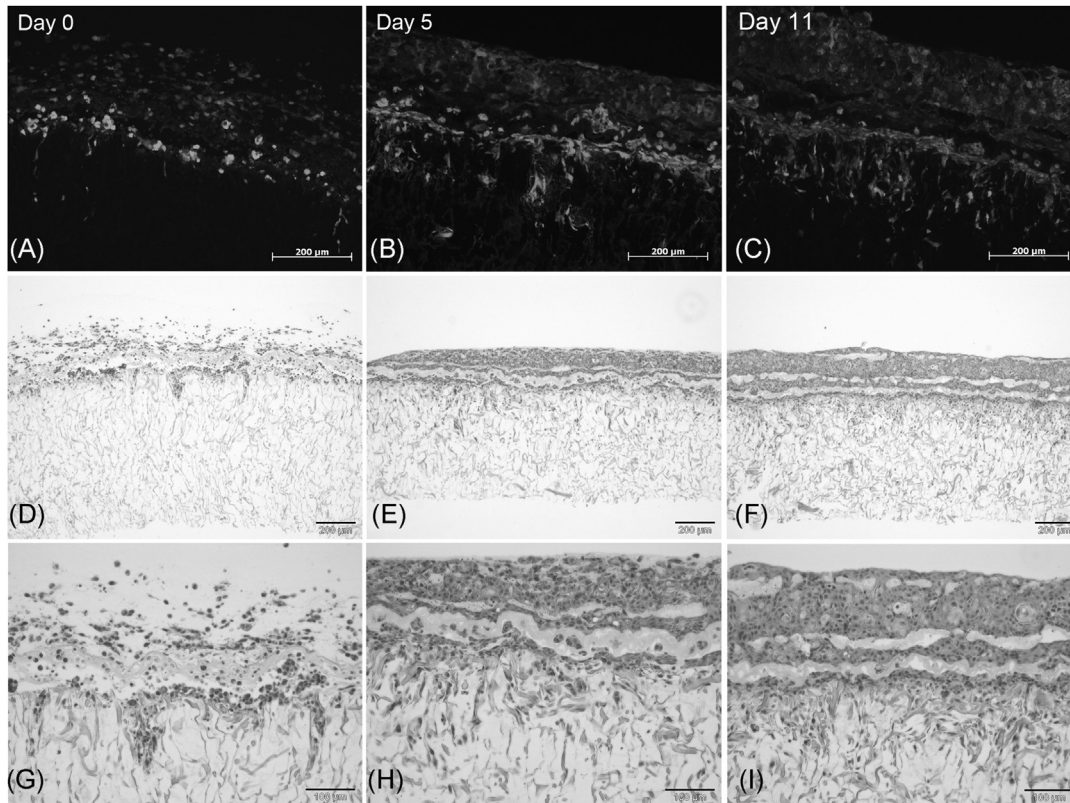
Skin constructs were cultured for 11 days and examined on day 0 (day after printing, when raised to the interface), day 5, and day 11. The used cell lines were stably transduced with genes for fluorescent molecules so that the cells could easily be detected via fluorescent microscopy at the end of the experiments.

On day 0, the cells were mostly rounded and still embedded in the collagen, which had served as the printing matrix (Fig. 15.5). On days 5 and 10, however, the keratinocytes were connected, forming an epidermis-like tissue. Analogously to the previous experiments, e-cadherin as a marker for adherens junctions could be detected in the epidermis-like layer on days 5 and 10, thereby confirming the formation of an epithelium.

In contrast to the keratinocytes, the printed fibroblasts partly migrated into the underlying Matrigel, following its fibers closely. Part of the cells stayed at the interface between the Matrigel and the keratinocytes, though. This is very promising for future *in vivo* applications.

In the Masson's trichrome staining inclusions of collagen could be seen in the epidermis, especially directly above the Matrigel. Probably, the printed cells—especially the fibroblasts—did not completely digest the collagen with which they were printed, but instead migrated out of it, leaving the collagen behind.

In summary, a bilayered skin construct was successfully created by laser-assisted bioprinting and subsequent *in vitro* culture.

**FIGURE 15.5**

Sections of the printed skin constructs, cultured *in vitro* at the air–liquid interface. Printed skin constructs were cultured at the air–liquid interface with differentiation medium for 11 days. Sections (A–C) show the printed cells, which directly exhibit fluorescence due to transduction (keratinocytes—red, fibroblasts—green), Masson's trichrome staining shows connective tissue in green and the cells in red. The fibroblasts start to migrate into the Matrigel already on day 1 (A, D, G) and continue to do so later on. The keratinocytes are rounded and not connected on day 1, but already form a dense tissue on day 5. The epidermis even increases in height until day 11. Scale bars represent 200 μm (A–F) and 100 μm (G–I).

Reprint from Michael, Sorg, Peck, Koch, et al. (2013).

15.3.3.4 Laser-Assisted Printing of Skin Tissue—In Vivo Culture

On their way to clinical applications, new therapies need to be tested in animal experiments to ensure the maximal safety possible. In the first step, often a rodent model is used, since these animals are small, simple to keep and breed, inexpensive, and different genetically altered strains are available. As Michael et al. used xenogeneic (keratinocytes) and allogeneic (fibroblasts) cells, they needed to use T-cell-deficient mice to avoid a rejection of the skin transplants (Michael, Sorg, Peck, Koch, et al., 2013). Analogously to the *in vitro* experiments, the used cell lines were stably transduced with genes for fluorescent molecules to enable direct detection of the printed cells.

For the *in vivo* evaluation, the dorsal skin fold chamber in mice was used. During the preparation of the chamber, the dorsal skin of the anaesthetized mouse was lifted and fixed via two titanium frames (chamber), resulting in a sandwich like construction. In one side, a full thickness wound was cut, while the opposite skin remained unharmed. The skin construct was now inserted into the wound. The chamber was closed by insertion of a glass cover slip, which was fixed with a snap ring. In contrast to humans, whose wounds heal by granulation tissue and re-epithelialization, wounds in rodents are mainly closed by contraction. In the chambers, the skin is fixed and no contraction of the wound area is possible. Furthermore, due to the cover slip, a continuous observation of the wound is possible and no change of dressings is needed. This reduces the stress for the animals immensely.

Skin constructs were printed analogously to the previously described ones and inserted into the chamber the day after printing (day 0). The *in vitro* culture over night gave the printed cells the opportunity to adhere to their surroundings. The animals were sacrificed after 11 days and analyzed by histology and immunohistochemistry.

At the end of the experiments, the skin constructs were tightly connected to the surrounding host tissue (Fig. 15.6). This macroscopic observation could be confirmed on the histological level. The printed cells were alive and could directly be tracked via fluorescence microscopy.

The printed keratinocytes formed a dense stratified tissue, similar to normal epidermis. In some samples, even a corneal layer could be observed (Fig. 15.7). The printed keratinocytes did not exhibit a complete differentiation, though, but a beginning one could be found (Fig. 15.8). Since the experiments only lasted 11 days and for a complete differentiation about 3 weeks are needed, these results are not unexpected.

Formation of adherens junctions and thereby tissue/epithelium formation was confirmed by detection of e-cadherin throughout the whole epithelium (Fig. 15.9). Despite the similarity to normal mouse skin, the epidermis developed by the printed keratinocytes was less thick and no rete ridges were present. This is not optimal for the mechanical stability of the skin, but promising, though, since it represents the first and successful step toward tissue engineering of skin by laser-assisted bioprinting. Thicker epithelium may be printed in future, including rete ridges.

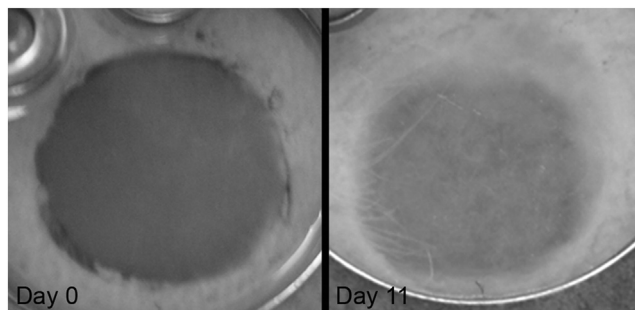
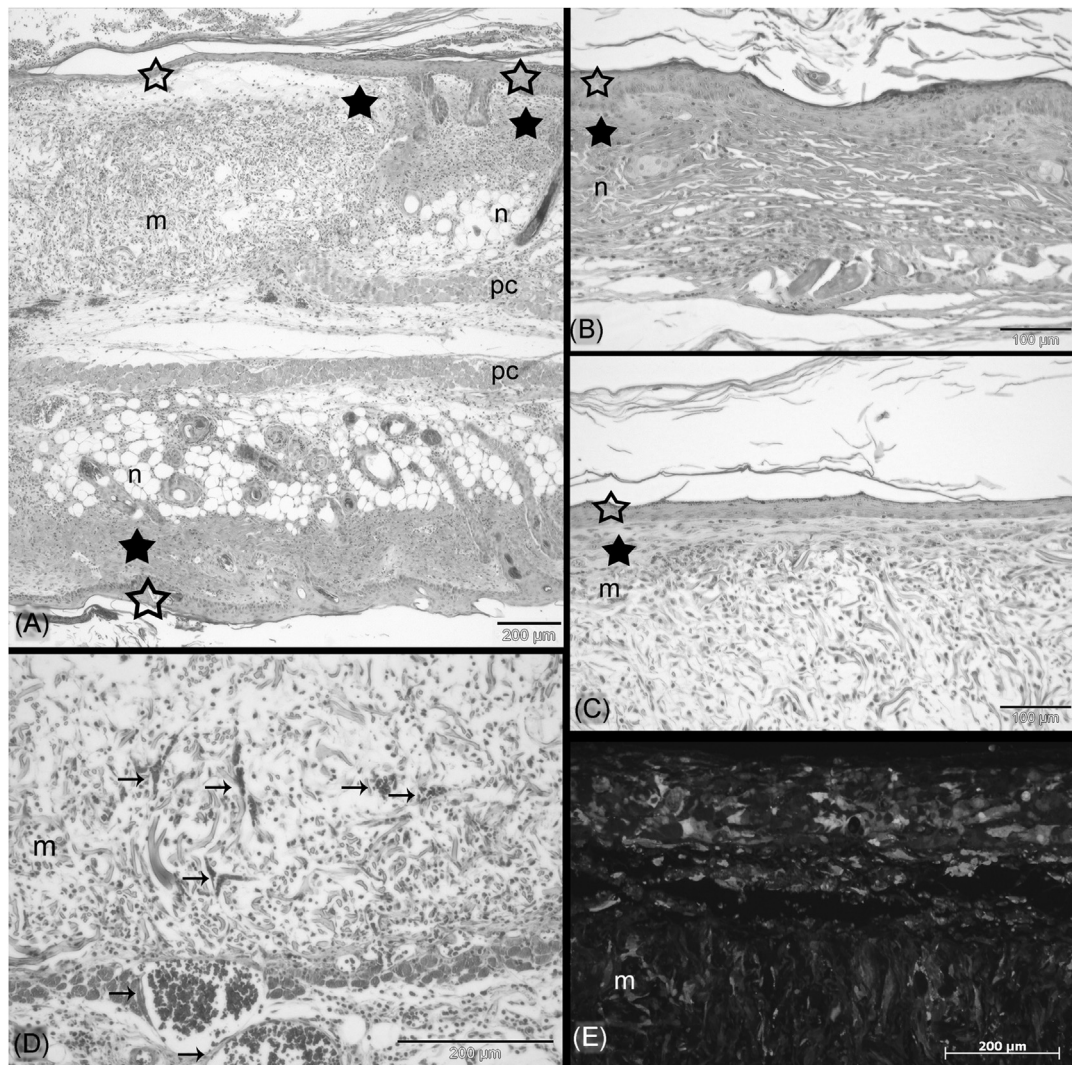


FIGURE 15.6

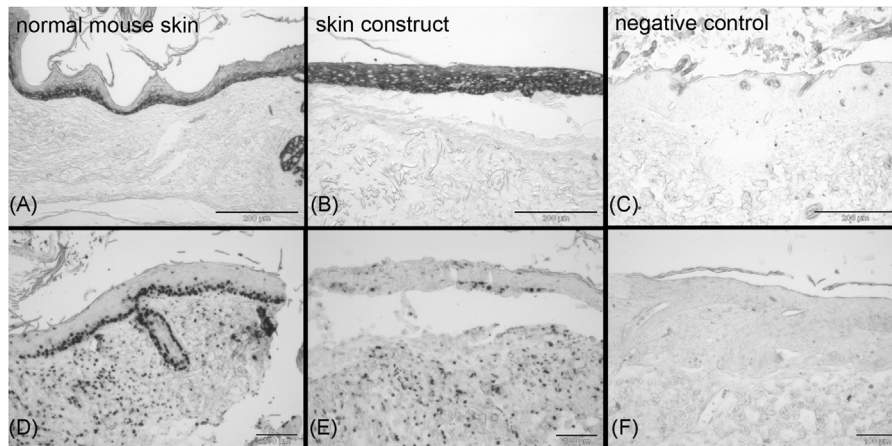
Printed skin constructs implanted in the dorsal skin fold chamber in nude mice. Printed skin constructs were inserted into full-thickness skin wounds in the dorsal skin of nude mice and fill the wounds completely after implantation (left). After 11 days, the constructs are connected to the surrounding host tissue (right).

Reprint from Michael, Sorg, Peck, Koch, et al. (2013).

**FIGURE 15.7**

Histological sections of the printed skin constructs after 11 days in the dorsal skin fold chambers. Printed skin constructs were implanted in the dorsal skin fold chamber in nude mice. Masson's trichrome staining (A–D) depicts connective tissue in green and cells in red. In an overview, the junction between the skin construct (m—Matriderm) and the surrounding native mouse skin (n) at the wound edge can be seen (A). The skin construct and the unharmed skin opposite from it are separated by the panniculus carnosus (pc). Both in the native mouse skin (B) and the printed skin (C) a dense epidermis (empty asterisks) and a corneal layer can be seen. The printed keratinocytes form the epidermis in the case of the printed skin constructs, as can be directly detected via their fluorescence (E, keratinocytes—green). The fibroblasts partly migrate into the Matriderm (E, fibroblasts—red). Collagen deposition is marked with filled asterisks. Blood vessels (arrows) are present in the printed skin constructs. Scale bars represent 100 μm (A, D, E) and 100 μm (B, C).

Reprint from Michael, Sorg, Peck, Koch, et al. (2013).

**FIGURE 15.8**

Detection of cytokeratin 14 and proliferation in printed skin constructs. Printed skin constructs were cultivated in the dorsal skin fold chamber in mice for 11 days. Cytokeratin 14 expression is only found in the suprabasal layers of the epidermis in native mouse skin (A). In contrast, it is present in the whole epidermis in the printed skin constructs. Proliferation (Ki67) is limited to the suprabasal layers in both normal mouse skin and the printed skin constructs (D, E). Scale bars represent 200 μm (A–C) and 100 μm (D–F).

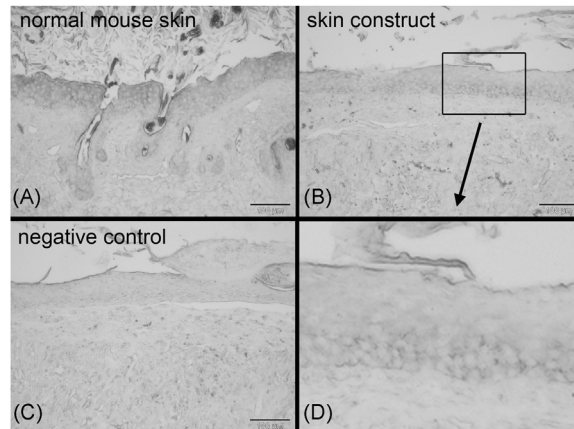
Reprint from Michael, Sorg, Peck, Koch, et al. (2013).

In contrast to the keratinocytes, only part of the printed fibroblasts remained on top of the Matrigel, while a large fraction migrated into it. Thereby, the fibroblasts and keratinocytes together formed a multilayered tissue (Fig. 15.7). This is very satisfactory, since an organotypic structure of the skin construct is essential for a future application.

Small blood vessels growing into the Matrigel from the depth of the wound bed and the wound edges could be found (Figs. 15.7 and 15.10). They were directed toward the printed cells, which may be explained by cytokine expression (e.g., vascular endothelium growth factor) of the cells (Ballaun, Weninger, Uthman, Weich, & Tschachler, 1995). In control experiments without cells, no vascularization of the constructs at all could be found (Michael, Sorg, Peck, Reimers, & Vogt, 2013). This strengthens the conclusion that the printed cells induced blood vessel formation.

15.3.4 INKJET-BASED *IN SITU* BIOPRINTED SKIN

Instead of printing skin and implanting it afterwards, Binder (2011) and Albanna et al. (2019) used a commercial inkjet printer to print skin cells directly into wounds *in situ*. He combined the printer with a laser scanning system to measure the wound size and calculate a wound surface model. Then the inkjet printer, mounted on a portable XYZ plotting system, was applied to fill the wound with hydrogel and cells. For the printing, they loaded each cell type into an individual cartridge

**FIGURE 15.9**

e-Cadherin expression detected by immunohistochemistry. Printed skin constructs were cultivated in the dorsal skin fold chamber in mice for 11 days. e-Cadherin expression can be detected in normal mouse skin (A) as well as in the skin constructs (B, D). Normal mouse skin without first antibody constitutes the negative control (C). All scale bars represent 100 μm .

Reprint from Michael, Sorg, Peck, Koch, et al. (2013).

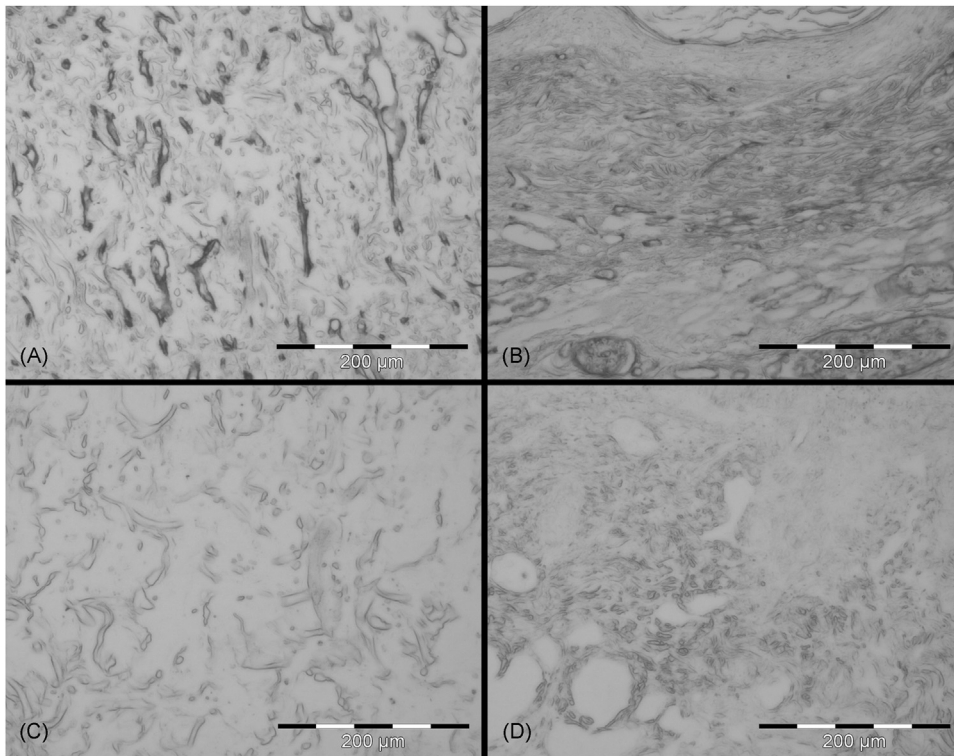
like color inks are contained in different cartridges. Thereby, fibroblasts and keratinocytes could be printed simultaneously.

With this printer, human skin cells were printed directly into a dorsal full-thickness skin wound on nude immunodeficient mice. For this, the cells were embedded in a hydrogel mixture of fibrinogen and collagen type I. After printing of each layer, the fibrinogen was cross-linked with printed or spray-coated thrombin.

It was demonstrated that the printed human cells remained in the mouse wound area. The printed cells accelerated the closure of the wound compared to printed hydrogel without cells by a factor of up to two. Within 3 weeks the printed skin cells developed a structure with organized dermal collagen and a formed epidermis. The printed human skin cells were still present in dermis and epidermis of the regenerated skin (Binder, 2011).

As a next step, the printer was used in a porcine wound model. Again, a full-thickness excisional wound was made on the dorsa. The wound was imaged with the laser scanning system and filled with fibroblasts and keratinocytes by printing. Here, the pigs were not immunodeficient, and autologous or allogeneic cells were used.

Again, the wounds treated with printed skin cells closed more quickly than the wounds treated with hydrogel alone. Two weeks after printing autologous skin cells, the keratinocytes established areas of epithelialization, which grew and covered the entire wound area within 8 weeks. An epidermis with the same thickness than the surrounding normal skin developed. A dermis–epidermis interface could be observed and the general composition of the printed skin appeared to be similar to native skin. Prelabeled printed skin cells were still visible in the center of the wound of one pig after 8 weeks.

**FIGURE 15.10**

Presence of blood vessels in the printed skin constructs. Printed skin constructs were cultivated in the dorsal skin fold chamber in mice for 11 days. Collagen VI expression (brown) indicates blood vessels. In the skin constructs, small vessels can be found in the Matrigel, reaching from the wound bed in the direction of the cells (A). Small and large vessels can be found in normal mouse skin (B). The respective negative controls are shown in C (Matrigel) and D (normal mouse tissue). Scale bars represent 200 μm each.

Reprint from Michael, Sorg, Peck, Koch, et al. (2013).

15.4 DISCUSSION OF THE DIFFERENT BIOPRINTING TECHNIQUES AND CLINICAL APPLICABILITY

15.4.1 OPTIMIZATION OF THE SKIN EQUIVALENTS

So far, different bioprinting techniques have been used to print fibroblast and keratinocyte skin cells in a bilayered 3D structure. The formation of tissue with intercellular junctions could be observed *in vitro* and *in vivo* (Koch et al., 2012; Michael, Sorg, Peck, Koch, et al., 2013). Implantation of *ex vivo* printed skin equivalents (Michael, Sorg, Peck, Koch, et al., 2013) and printing skin cells in wounds *in situ* (Binder, 2011), both result in an ingrowth of the printed tissue into the surrounding natural skin. Thus the printed skin is capable of covering wounds for preventing liquid or protein loss or infections.

However, these skin equivalents lack other important functions of natural skin, such as an effective barrier function, the regulation of the body temperature by sweat glands or the immune competence. Also, the printed skin's function as a sensory organ is at least very constricted. Furthermore, the appearance of the printed skin differs fundamentally from natural skin and no hair was included so far. Moreover, a high mechanical stability of the printed skin equivalents is needed. Perhaps, some of these functions might be regenerated by immigrating cells from the patient's organism.

Nevertheless, the skin constructs need improvement by adding further cell types like endothelial cells for quick blood vessel generation, hair follicle cells, melanocytes for pigmentation, immune cells (Langerhans cells, macrophages, leukocytes, plasma cells, and mast cells), nerve and Schwann cells for perception, or cells present in perspiratory glands to enable sweating and thereby temperature homeostasis. However, just adding these cell types will probably not suffice to create all of the necessary cutaneous appendages. Instead, the correct microenvironments need to be created to enable the different cell types to fulfill their destinations.

Bioprinting offers the possibility to exactly deposit each cell type at its correct 3D location, helping to create the necessary microenvironment for the cells to fulfill their functions. This makes this technique such a promising tool for tissue engineering. While several bioprinting setups, commercially available or individually constructed, are used for tissue engineering research, they do not fulfill the requirements for clinical applications in skin tissue generation, yet. For the use in clinical skin reconstruction, the printing needs to be safe, rapid, and cost effective. Thus several adaptations are needed on the way from bench to bedside and different challenges need to be met.

15.4.2 TECHNICAL AND BIOMEDICAL CHALLENGES

A scale-up of the printed skin substitutes is necessary, especially with regard to large burn injuries. This is partly a technical challenge. Actually, the printing area of most printers is too small and the printing velocity of several printers is too low, since often, these printers are not throughput-optimized, yet. While scaling up the printing area is just an engineering task, there will be fundamental limits for the printing velocity. Especially, printing time usually increases with the cube of the printing resolution. Parallel printing with multiple printheads may increase the printing velocity further on toward a high throughput system. However, a scale-up is also a biological challenge, since a great many of skin cells are needed for large burn wounds. Thus appropriate cell sources and culture conditions are necessary.

For the production, storage, and delivery of the printed skin substitutes, high standards of quality and safety need to be applied, usually good manufacturing practice (GMP) conditions or similar. The according guidelines include an adequate quality management and quality control (including self-inspection), appropriately trained personnel, suitable premises and equipment, and an extensive documentation of all steps of the manufacture, storage and distribution of the products ([International Society for Pharmaceutical Engineering, 2014](#)). Therefore concerning the printing setups, vast technical adaptations need to be implemented to reach GMP standard. This includes, for example, the encapsulation of the printing area into a laminar flow hood to ensure sterility. Also, as many of the components used in the printing process and the cell culture as possible should be disposable to minimize contamination ([Chang, Boland, Williams, & Hoying, 2011](#)).

To avoid an infection of the cells and the skin tissues, an integration of the different process steps (isolation of cells from a biopsy, if required; cell culture, bioprinting, and the tissue culture)

in one sterile environment would be advantageous. [Walles et al. \(2014\)](#) established an integrated system without a bioprinting system. This “tissue factory” is capable of producing up to 5000 tissue pieces per month fully automated.

15.4.3 STEM CELLS AS POSSIBLE CELL SOURCES FOR BIOPRINTING OF SKIN

Additionally to the technical challenges, the question about the source of the printed cells arises. Ideally, autologous cells should be used to avoid rejection and transmission of (viral) diseases. Although it is quite feasible to propagate enough cells from a skin biopsy prior to a planned surgical intervention of, for example, a small chronic wound, in the case of extensive burn injuries, donor skin for biopsies to gain autologous cells is scarce. Therefore other cell sources are needed. In this context, the use of stem cells has been proposed. They are abundantly present in the different organs, since most tissue is capable of self-renewal ([Metcalf & Ferguson, 2008](#)). The use of adult stem cells is not restricted by ethical considerations, and adult stem cells can be found in, for example, bone marrow, adipose tissue, blood, and skin. Especially stem cells derived from adipose tissue (ASCs) are very advantageous as they are easy to isolate and propagate, and are of bountiful occurrence in humans ([Zuk et al., 2001](#)). Apart from their differentiation to bone, cartilage, and fat, they can also be differentiated to endothelial cells to promote quick vascularization of skin substitutes ([Auxenfans et al., 2011](#)). Moreover, stem cells from sweat glands are easy to gain and to propagate, and enhance vascularization, too ([Danner et al., 2012](#)).

As an alternative to print skin tissue, [Skardal et al. \(2012\)](#) printed stem cells (amniotic fluid-derived stem cells and bone marrow-derived mesenchymal stem cells (MSCs), embedded in fibrin-collagen gel) in dorsal full-thickness excisional wounds in mice. They demonstrated that these cells considerably stimulate the regeneration of the skin by the mouse organism. Wound closure and re-epithelialization were significantly greater than closure and re-epithelialization in wounds treated by fibrin-collagen gel only. Higher microvessel density, bigger capillary diameters, and thicker epithelial tissue were observed by applying these stem cells. However, the stem cells did not integrate into the skin tissue, but promote the skin tissue regeneration by secreted trophic factors.

The use of stem cells might be dangerous, though, since stem cells might differentiate into harmful cells or may secrete factors that enhance formation and growth of cancer cells. In this context, the interaction between the tumor cells and their microenvironment is crucial for tumor development, and stem cells may have a large influence on it. On the other hand, MSCs are recruited to and accumulate in tumors. Therefore they contribute to the angiogenesis of the tumors and suppress antitumor immune responses ([Cuiffo & Karnoub, 2012](#)). Furthermore, it could be shown that cancer cells are able to induce malignant transformation of MSCs *in vitro* via paracrine effects ([Liu, Zhang, Bai, Cui, & Zhu, 2012](#)).

Generally, the risk of tumor formation depends on the origin of the stem cells, the extent of their *ex vivo* expansion, the induced differentiation, and the site/route of administration. Differences can also be found between embryonic stem cells, which are pluripotent, and adult stem cells, which are multipotent. Therefore utmost care has to be exercised concerning the choice of the used cells in tissue engineering.

Even if stem cells were safe enough to use, the question arises if the cells should be used in their stem cell phenotype—as a “growth factor factory”—or if they should be differentiated to skin cells before implantation. And when keratinocytes are used, can they be used in their

undifferentiated form, or do they need to be differentiated *in vitro* to form an epithelium before implantation? This cannot be answered completely, yet. Often undifferentiated stem cells are employed to utilize their regenerative potential. But these might be involved in tumor formation, as described above. Concerning the keratinocytes, Koch et al. (2012) and Michael, Sorg, Peck, Koch, et al. (2013) used nondifferentiated cells for the bioprinting and implanted them also in a nondifferentiated state. In the animals, they could detect a beginning differentiation. This means that the body with its growth factors could be enough to provide a suitable environment for the differentiation of the cells and the formation of a stable epithelium.

To have enough skin cells available quickly in the event of severe burn injuries, it would of course be better if no autologous cells were necessary, but rather if cells are available that could be used in many patients. As already mentioned, this would normally lead to rejection reactions. However, there are research approaches to overcome this. According to Schlottmann, Strauss, Hake, Vogt, and Bucan (2019), allogeneous nonimmunogenic cells might be an alternative to autologous transplants. To overcome rejection of allografts, immunogenicity needs to be reduced. Many viruses have developed suitable strategies to interfere with immune recognition. The human cytomegalovirus suppresses the viral peptide presentation by reducing MHC-I expression on the cell surface of infected cells, and thus the immune response of the infected organism. In frame a proof of concept study, the group demonstrated a downregulation of MHC-I expression using viral vectors as carrier for the United States11 gene of human cytomegalovirus. Human primary keratinocytes transiently transfected with the carrier displayed a downregulation of MHC-I after 24 hours (Schlottmann et al., 2019). Using a stable transfection system, a permanent downregulation might be achieved, representing a step toward an immune-privileged transplant. As cultivation matrix, Schlottmann et al. used Matrigel that was also applied by Koch and Michael for cell printing. Histological assessment demonstrated long-term survival of United States11 transfected cells as well as patterns comparable to epidermal layers of human skin (Schlottmann et al., 2019). Therefore a successful combination of both basic approaches seems to be realistic.

15.5 Conclusion

Bioprinting bears the promise to “build” a skin in its full complexity, albeit the already printed skin tissue is far from its natural archetype and lacks most of its functions. The possibility of transferring biomaterial, including cells, macromolecules, and growth factors, to exactly the desired 3D position in a (skin) tissue is the unique feature of bioprinting and its major strength. The different printing technologies might meet the requirements for a clinical or commercial application (high throughput, high resolution, epithelium-like high cell density, and a mechanical stable extracellular matrix) to a different degree. However, all of these techniques have further potential for improvement. Skin is particularly suitable for improving bioprinting technologies. Starting with only the two major cell types in skin, fibroblasts and keratinocytes, tissue was already printed. The complexity can be increased gradually by integrating further cell types. Thus the missing functions may be added bit-by-bit.

The functional and esthetical enhancement of the printed tissue should be the focus of future research, since the printed skin equivalents are still inchoate. The integration of a vascular network is one of the major challenges but it would accelerate wound healing and would be essential, since

insufficient vascularization often leads to the rejection of transplants, especially very large ones. Moreover, printing skin with a connectable vascular network would enable further applications in drug testing, for example, in a body-on-a-chip system. First steps toward a printed vascular network, applied *in vivo*, were already done (e.g., Gaebel et al., 2011).

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References

- Albanna, M., Binder, K. W., Murphy, S. V., Kim, J., Qasem, S. A., Zhao, W., . . . Yoo, J. J. (2019). In situ bioprinting of autologous skin cells accelerates wound healing of extensive excisional full-thickness wounds. *Scientific Reports*, 9, 1856.
- Auxenfans, C., Lequeux, C., Perrusel, E., Mojallal, A., Kinikoglu, B., & Damour, O. (2011). Adipose-derived stem cells (ASCs) as a source of endothelial cells in the reconstruction of endothelialized skin equivalents. *Journal of Tissue Engineering and Regenerative Medicine*, 6(7), 512–518.
- Ballaun, C., Weninger, W., Uthman, A., Weich, H., & Tschachler, E. (1995). Human keratinocytes express the three major splice forms of vascular endothelial growth factor. *The Journal of Investigative Dermatology*, 104(1), 7–10.
- Barron, J. A., Krizman, D. B., & Ringeisen, B. R. (2005). Laser printing of single cells: Statistical analysis, cell viability, and stress. *Annals of Biomedical Engineering*, 33(2), 121–130.
- Barron, J. A., Ringeisen, B. R., Kim, H., Spargo, B. J., & Chrisey, D. B. (2004). Application of laser printing to mammalian cells. *Thin Solid Films*, 453–454, 383–387.
- Begandt, D., Bintig, W., Oberheide, K., Schlie, S., & Ngezahayo, A. (2010). Dipyrriamole increases gap junction coupling in bovine GM-7373 aortic endothelial cells by a cAMP-protein kinase A dependent pathway. *Journal of Bioenergetics and Biomembranes*, 42, 79.
- Bellows, C. G., Melcher, A. H., & Aubin, J. E. (1982). Association between tension and orientation of periodontal ligament fibroblasts and exogenous collagen fibres in collagen gels in vitro. *Journal of Cell Science*, 58, 125–138.
- Berner, R., Asselineau, D., Vioux, C., Chevallier-Lagente, O., Bouadjar, B., Sarasin, A., & Magnaldo, T. (2001). Clues to epidermal cancer proneness revealed by reconstruction of DNA repair-deficient xeroderma pigmentosum skin in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, 98(14), 7817–7822.
- Bigelow, R. L. H., Jen, E. Y., Delehedde, M., Chari, N. S., & McDonnell, T. J. (2005). Sonic hedgehog induces epidermal growth factor dependent matrix infiltration in HaCaT keratinocytes. *Journal of Investigative Dermatology*, 124, 457.
- Binder, K. W. (2011). *In situ bioprinting of the skin. A thesis submitted in partial fulfillment of the requirements of Wake Forest University for the Degree of Doctor of Philosophy.* Winston-Salem, NC: Wake Forest University.

- Boehnke, K., Mirancea, N., Pavesio, A., Fusenig, N. E., Boukamp, P., & Stark, H. J. (2007). Effects of fibroblasts and microenvironment on epidermal regeneration and tissue function in long-term skin equivalents. *European Journal of Cell Biology*, 86, 731.
- Born, C., Zhang, Z., Al-Rubeai, M., & Thomas, C. R. (1992). Estimation of disruption of animal cells by laminar shear stress. *Biotechnology and Bioengineering*, 40, 1004.
- Chang, C. C., Boland, E. D., Williams, S. K., & Hoying, J. B. (2011). Direct-write bioprinting three-dimensional biohybrid systems for future regenerative therapies. *Journal of Biomedical Materials Research, Part B: Applied Biomaterials*, 98(1), 160–170.
- Chang, R., Nam, J., & Sun, W. (2008). Effects of dispensing pressure and nozzle diameter on cell survival from solid freeform fabrication-based direct cell writing. *Tissue Engineering Part A*, 14, 41.
- Cubo, N., Garcia, M., del Cañizo, J. F., Velasco, D., & Jorcano, J. L. (2016). 3D bioprinting of functional human skin: Production and in vivo analysis. *Biofabrication*, 9, 015006.
- Cuiffo, B. G., & Karnoub, A. E. (2012). Mesenchymal stem cells in tumor development: Emerging roles and concepts. *Cell Adhesion and Migration*, 6(3), 220–230.
- Danner, S., Kremer, M., Petschnik, A. E., Nagel, S., Zhang, Z., Hopfner, U., ... Egaña, J. T. (2012). The use of human sweat gland-derived stem cells for enhancing vascularization during dermal regeneration. *Journal of Investigative Dermatology*, 132(6), 1707–1716.
- Delehedde, M., Cho, S. H., Hamm, R., Brisbay, S., Ananthaswamy, H. N., Kripke, M., & McDonnell, T. J. (2001). Impact of Bcl-2 and Ha-ras on keratinocytes in organotypic culture. *The Journal of Investigative Dermatology*, 116, 366.
- Duocastella, M., Fernández-Pradas, J. M., Morenza, J. L., & Serra, P. (2009). Time-resolved imaging of the laser forward transfer of liquids. *Journal of Applied Physics*, 106, 084907.
- Eckert, R. L., Sturniolo, M. T., Broome, A.-M., Ruse, M., & Rorke, E. A. (2005). Transglutaminase function in epidermis. *Journal of Investigative Dermatology*, 124(3), 481–492.
- Fitzgerald, D. J., Fusenig, N. E., Boukamp, P., Piccoli, C., Mesnil, M., & Yamasaki, H. (1994). Expression and function of connexin in normal and transformed human keratinocytes in culture. *Carcinogenesis*, 15, 1859.
- Fransson, J. (2000). Tumour necrosis factor-alpha does not influence proliferation and differentiation of healthy and psoriatic keratinocytes in a skin-equivalent model. *Acta Dermato-Venereologica*, 80(6), 416–420.
- Gaebel, R., Ma, N., Liu, J., Guan, J., Koch, L., Klopsch, C., ... Steinhoff, G. (2011). Patterning human stem cells and endothelial cells with laser printing for cardiac regeneration. *Biomaterials*, 32, 9218–9230.
- Gibbs, S. (2009). In vitro irritation models and immune reactions. *Skin Pharmacology and Physiology*, 22(2), 103–113.
- Golinski, P. A., Zöller, N., Kippenberger, S., Menke, H., Bereiter-Hahn, J., & Bernd, A. (2009). Development of an engraftable skin equivalent based on matrigel with human keratinocytes and fibroblasts. *Handchirurgie, Mikrochirurgie, Plastische Chirurgie: Organ der Deutschsprachigen Arbeitsgemeinschaft für Handchirurgie: Organ der Deutschsprachigen Arbeitsgemeinschaft für Mikrochirurgie der Peripheren Nerven und Gefäße: Organ der V*, 41(6), 327.
- Gruene, M., Deiwick, A., Koch, L., Schlie, S., Unger, C., Hofmann, N., ... Chichkov, B. (2011). Laser printing of stem cells for biofabrication of scaffold-free autologous grafts. *Tissue Engineering Part C, Methods*, 17, 79–87.
- Gruene, M., Pflaum, M., Deiwick, A., Koch, L., Schlie, S., Unger, C., ... Chichkov, B. (2011). Adipogenic differentiation of laser-printed 3D tissue grafts consisting of human adipose-derived stem cells. *Biofabrication*, 3, 015005.
- Gruene, M., Unger, C., Koch, L., Deiwick, A., & Chichkov, B. (2011). Dispensing pico to nanolitre of a natural hydrogel by laser-assisted bioprinting. *Biomedical Engineering Online*, 10, 19.

- Guillotin, B., Souquet, A., Catros, S., Duocastella, M., Pippenger, B., Bellance, S., ... Guillemot, F. (2010). Laser assisted bioprinting of engineered tissue with high cell density and microscale organization. *Biomaterials*, 31, 7250–7256.
- Gumbiner, B. M. (1996). Cell adhesion: The molecular basis of tissue architecture and morphogenesis. *Cell*, 84, 345.
- Herndon, D. N. (2002). *Total burn care* (2nd ed.). Saunders W.B.
- Hopp, B., Smausz, T., Kresz, N., Barna, N., Bor, Z., Kolozsvari, L., ... Nogradi, A. (2005). Survival and proliferative ability of various living cell types after laser-induced forward transfer. *Tissue Engineering*, 11 (11–12), 1817–1823.
- Houben, E., De Paepe, K., & Rogiers, V. (2007). A keratinocyte's course of life. *Skin Pharmacology and Physiology*, 20(3), 122–132.
- International Society for Pharmaceutical Engineering. (2014). *What is GMP [Online]*. Retrieved April 26, 2014, from <http://www.ispe.org/gmp-resources/what-is-gmp>.
- Jorgensen, A. M., Varkey, M., Gorkun, A., Clouse, C., Xu, L., Chou, Z., ... Atala, A. (2020). Bioprinted skin recapitulates normal collagen remodeling in full-thickness wounds. *Tissue Engineering: Part A*, 26(9–10), 512–526.
- Kataoka, T., Umeda, M., Shigeta, T., Takahashi, H., & Komori, T. (2010). A new in vitro model of cancer invasion using AlloDerm® a human cadaveric dermal equivalent: A preliminary report. *Kobe Journal of Medical Sciences*, 55(5), E106–E115.
- Khalil, S., Nam, J., & Sun, W. (2005). Multi-nozzle deposition for construction of 3D biopolymer tissue scaffolds. *Rapid Prototyping Journal*, 11, 9.
- Ko, K., Arora, P., Lee, W., & McCulloch, C. (2000). Biochemical and functional characterization of intercellular adhesion and gap junctions in fibroblasts. *American Journal of Physiology – Cell Physiology*, 279, C147–C157.
- Koch, L., Deiwick, A., Schlie, S., Michael, S., Gruene, M., Coger, V., ... Chichkov, B. (2012). Skin tissue generation by laser cell printing. *Biotechnology and Bioengineering*, 109, 1855–1863.
- Koch, L., Kuhn, S., Sorg, H., Gruene, M., Schlie, S., Gaebel, R., ... Chichkov, B. (2010). Laser printing of skin cells and human stem cells. *Tissue Engineering Part C, Methods*, 16, 847–854.
- Koster, M. I. (2009). Making an epidermis. *Annals of the New York Academy of Science*, 1170, 7–10.
- Kuhn, S., Radtke, C., Allmeling, C., Vogt, P. M., & Reimers, K. (2011). Keratinocyte culture techniques in medical and scientific applications. In U. Khopkar (Ed.), *Skin biopsy – Perspectives*. InTech.
- Lee, W., Debasitis, J. C., Lee, V. K., Lee, J.-H., Fischer, K., Edminster, K., ... Yoo, S.-S. (2009). Multi-layered culture of human skin fibroblasts and keratinocytes through three-dimensional freeform fabrication. *Biomaterials*, 30, 1587.
- Lin, Y., Huang, Y., & Chrisey, D. B. (2009). Droplet formation in matrix-assisted pulsed-laser evaporation direct writing of glycerol-water solution. *Journal of Applied Physics*, 105(9), 093111.
- Lin, Y., Huang, Y., & Chrisey, D. B. (2011). Metallic foil-assisted laser cell printing. *Journal of Biomechanical Engineering*, 133, 025001.
- Linge, C. (2004). Establishment and maintenance of normal human keratinocyte cultures. In J. Picot (Ed.), *Methods in molecular medicine, Vol. 107: Human cell culture protocols* (2nd ed, p. 1). Totowa, NJ: Humana Press Inc.
- Liu, J., Zhang, Y., Bai, L., Cui, X., & Zhu, J. (2012). Rat bone marrow mesenchymal stem cells undergo malignant transformation via indirect co-cultured with tumour cells. *Cell Biochemistry and Function*, 30 (8), 650–656.
- Liu, N., Huang, S., Yao, B., Xie, J., Wu, X., & Fu, X. (2016). 3D bioprinting matrices with controlled pore structure and release function guide in vitro self-organization of sweat gland. *Scientific Reports*, 6, 34410.
- Mertsching, H., Weimer, M., Kersen, S., & Brunner, H. (2008). Human skin equivalent as an alternative to animal testing. *GMS Krankenhaushygiene interdisziplinär*, 3(1), ISSN: 1863-5245.

- Mese, G., Richard, G., & White, T. W. (2007). Gap junctions: Basic structure and function. *Journal of Investigative Dermatology*, 127, 2516–2524.
- Metcalfe, A. D., & Ferguson, M. W. (2008). Skin stem and progenitor cells: Using regeneration as a tissue-engineering strategy. *Cellular and Molecular Life Sciences*, 65(1), 24–32.
- Michael, S. (2013). *Laser induced forward transfer of biomaterials to create a skin substitute for burn patients* (PhD thesis). Gottfried Wilhelm Leibniz Universität, Hannover, Germany.
- Michael, S., Sorg, H., Peck, C.-T., Koch, L., Deiwick, A., Chichkov, B., ... Reimers, K. (2013). Tissue engineered skin substitutes created by laser-assisted bioprinting form skin-like structures in the dorsal skin fold chamber in mice. *PLoS One*, 8(3), e57741.
- Michael, S., Sorg, H., Peck, C.-T., Reimers, K., & Vogt, P. M. (2013). The mouse dorsal skin fold chamber as a means for the analysis of tissue engineered skin. *Burns: Journal of the International Society for Burn Injuries*, 39(1), 82–88.
- Morritt, A. N., Bortolotto, S. K., Dilley, R. J., Han, X. L., Kompa, A. R., McCombe, D., ... Morrison, W. A. (2007). Cardiac tissue engineering in an in vivo vascularized chamber. *Circulation*, 115, 353–360.
- Niessen, C. M. (2007). Tight junctions/adherens junctions: Basic structure and function. *Journal of Investigative Dermatology*, 127, 2525–2532.
- Okazaki, M., Suzuki, Y., Yoshimura, K., & Harii, K. (2005). Construction of pigmented skin equivalent and its application to the study of congenital disorders of pigmentation. *Scandinavian Journal of Plastic and Reconstructive Surgery and Hand Surgery*, 39(6), 339–343.
- Okeke, D. N., Tsuboi, R., & Ogawa, H. (2001). Quantification of *Candida albicans* actin mRNA by the LightCycler system as a means of assessing viability in a model of cutaneous candidiasis. *Journal of Clinical Microbiology*, 39(10), 3491–3494.
- Ovsianikov, A., Gruene, M., Pflaum, M., Koch, L., Maiorana, F., Wilhelmi, M., ... Chichkov, B. (2010). Laser printing of cells into 3D scaffolds. *Biofabrication*, 2, 014104.
- Pallua, N., & von Bülow, S. (2006). Methods of burn treatment. Part II: Technical aspects – Behandlungskonzepte bei Verbrenungen. *Der Chirurg; Zeitschrift für Alle Gebiete der Operativen Medizen*, 77(2), 179–186.
- Proksch, E., Brandner, J. M., & Jensen, J. M. (2008). The skin: An indispensable barrier. *Experimental Dermatology*, 17(12), 1063–1072.
- Richard, G. (2000). Connexins: A connection with the skin. *Experimental Dermatology*, 9, 77.
- Riedel, K., Ryssel, H., Koellensperger, E., Germann, G., & Kremer, T. (2008). Pathogenesis of chronic wounds – Pathophysiologie der chronischen Wunde. *Der Chirurg; Zeitschrift für Alle Gebiete der Operativen Medizen*, 79(6), 526–534.
- Ringeisen, B. R., Kim, H., Barron, J. A., Krizman, D. B., Chrisey, D. B., Jackman, S., ... Spargo, B. J. (2004). Laser printing of pluripotent embryonal carcinoma cells. *Tissue Engineering*, 10(3–4), 483–491.
- Schiele, N. R., Chrisey, D. B., & Corr, D. T. (2011). Gelatin-based laser direct-write technique for the precise spatial patterning of cells. *Tissue Engineering Part C, Methods*, 17(3), 289–298.
- Schiele, N. R., Corr, D. T., Huang, Y., Raof, N. A., Xie, Y., & Chrisey, D. B. (2010). Laser-based direct-write techniques for cell printing. *Biofabrication*, 2, 032001.
- Schlie, S., Mazur, K., Bintig, W., & Ngezahayo, A. (2010). Cell cycle dependent regulation of gap junction coupling and apoptosis in GFSHR-17 granulosa cells. *Journal of Biomedical Science and Engineering*, 3, 884–891.
- Schlottmann, F., Strauss, S., Hake, K., Vogt, P. M., & Bucan, V. (2019). Down-regulation of MHC class I expression in human keratinocytes using viral vectors containing United States11 gene of human cytomegalovirus and cultivation on bovine collagen-elastin matrix (Matriderm®): Potential approach for an immune-privileged skin substitute. *International Journal of Molecular Sciences*, 20(9), 2056.

- Schoop, V. M., Mirancea, N., & Fusenig, N. E. (1999). Epidermal organization and differentiation of HaCaT keratinocytes in organotypic coculture with human dermal fibroblasts. *Journal of Investigative Dermatology*, 112(3), 343.
- Simon, A. M., & Goodenough, D. A. (1998). Diverse functions of vertebrate gap junctions. *Trends in Cell Biology*, 8, 477.
- Skardal, A., Mack, D., Kapetanovic, E., Atala, A., Jackson, J. D., Yoo, J., & Soker, S. (2012). Bioprinted amniotic fluid-derived stem cells accelerate healing of large skin wounds. *Stem Cells Translational Medicine*, 1, 792–802.
- Unger, C., Gruene, M., Koch, L., Koch, J., & Chichkov, B. (2011). Time-resolved imaging of hydrogel printing via laser-induced forward transfer. *Applied Physics A*, 103, 271–277.
- Walles H., et al. (2014). *Information from conversation and online*. <http://www.tissue-factory.com> (Accessed April 25, 2014).
- Wiszniewski, L., Limat, A., Saurat, J.-H., Meda, P., & Salomon, D. (2000). Differential expression of connexins during stratification of human keratinocytes. *The Journal of Investigative Dermatology*, 115, 278.
- Xie, Y., Rizzi, S. C., Dawson, R., Lynam, E., Richards, S., Leavesley, D. I., & Upton, Z. (2010). Development of a three-dimensional human skin equivalent wound model for investigating novel wound healing therapies. *Tissue Engineering Part C, Methods*, 16(5), 1111–1123.
- Yan, J., Huang, Y., & Chrisey, D. B. (2013). Laser-assisted printing of alginate long tubes and annular constructs. *Biofabrication*, 5, 015002.
- Zuk, P. A., Zhu, M., Mizuno, H., Huang, J., Futrell, J. W., Katz, A. J., . . . Hedrick, M. H. (2001). Multilineage cells from human adipose tissue: Implications for cell-based therapies. *Tissue Engineering*, 7(2), 211–228.

Nanotechnology and 3D/4D Bioprinting for Neural Tissue Regeneration

16

Wei Zhu, Nathan J. Castro, Yin-Lin Shen and Lijie Grace Zhang

Department of Mechanical and Aerospace Engineering, The George Washington University, Washington, DC, United States

16.1 INTRODUCTION

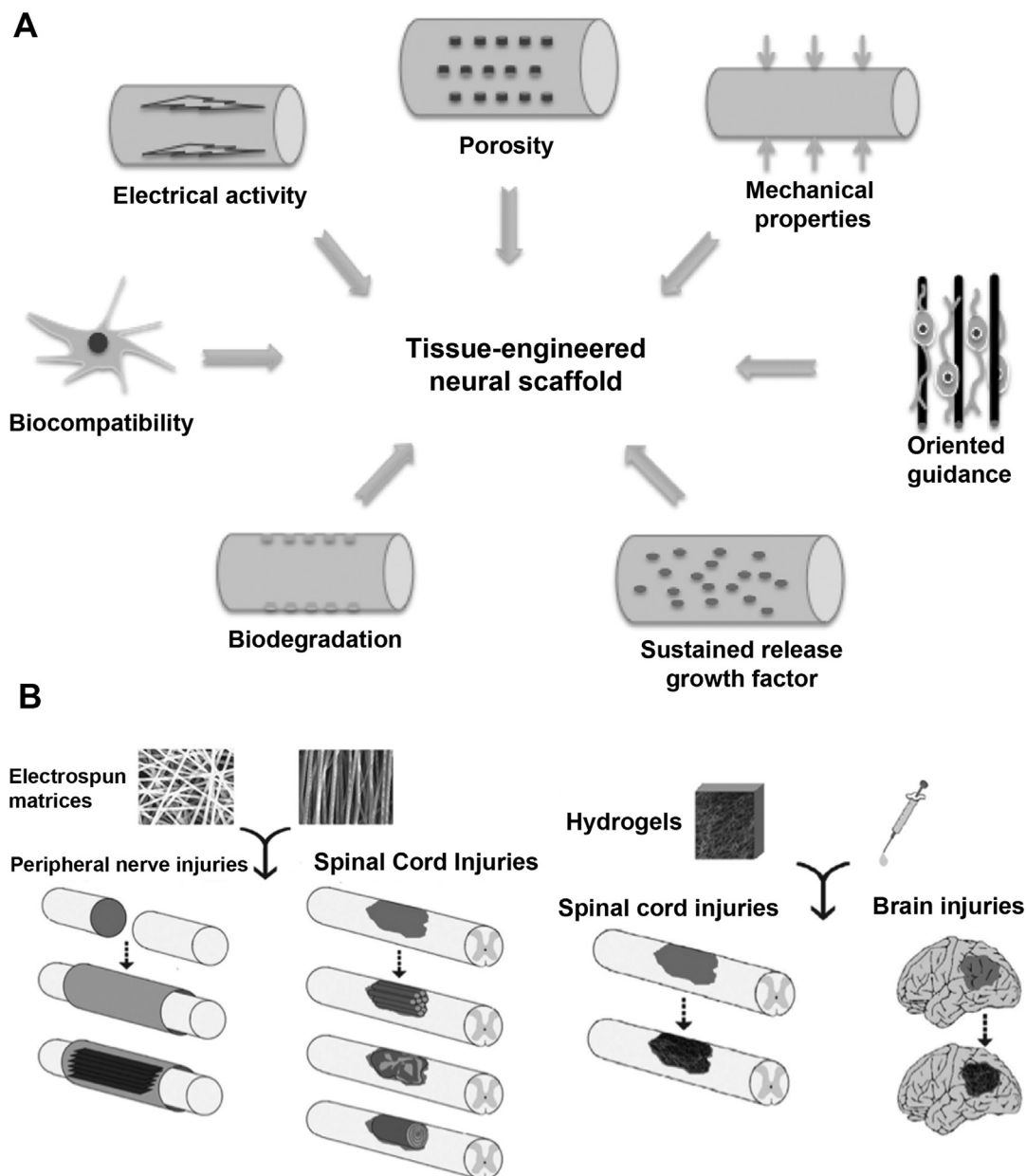
Acute traumatic injuries to, and debilitating chronic degenerative diseases of, the nervous system typically lead to the loss of central nervous system (CNS) and peripheral nervous system (PNS) function. Traumatic injury of the CNS may include traumatic brain injuries (TBIs) and spinal cord injuries (SCIs) (Shoichet, Tate, Douglas Baumann, & Laplaca, 2008), which have had a severe impact on the national healthcare system and the overall quality of life of patients around the world. It is estimated that there are approximately 69 million individuals globally are suffering TBI from all causes each year (Michael et al., 2019). In spite of the development of numerous clinical treatments, therapeutics for successfully bridging injured nerve gaps and full recovering of neural function are still in their infancy thus leading scientists to draw inspiration from neural tissue engineering strategies in the repair of CNS and PNS injuries (Constans, 2004; Shoichet & Schmidt, 2001).

Neural tissue engineering is focused on the development of biological substitutes that integrate biomimetic scaffolding with cells for the restoration, maintenance, and improvement of neural tissue function. As critical components in successful nerve regeneration, 3D scaffolds and scaffolding materials provide a necessary physical support to facilitate cell function resulting in better host tissue engraftment and subsequent new tissue development. Ideally, a tissue engineered nerve scaffold should meet the following criteria: (1) biocompatibility, the scaffold should promote cell adhesion, proliferation, and differentiation in the absence of immune and cytotoxic response; (2) biodegradability, the scaffold should degrade at a rate closely matching the formation of new tissue and eventually expelled from the body; (3) conductivity, neural communication depends on the action potential produced at the synapse. The electrical conductivity of scaffolds can thereby promote neurite outgrowth and nerve regeneration; (4) suitable mechanical properties, the scaffolds must not increase tension in the lesion site or collapse during regular movement; and (5) porous interconnectivity, a porous structure that mimics the extracellular matrix (ECM) of native tissue allowing for good spatial distribution of cells and exchange of nutrients and waste (Cunha, Panseri, & Antonini, 2011; Subramanian, Krishnan, & Sethuraman, 2009). Additionally, it is encouraged to incorporate

morphological and chemical features for improved axonal guidance of the tissue in an effort to bridge discontinuous injuries and facilitate nerve regeneration. Fig. 16.1 illustrates the key features that an ideal neural scaffold should have and several typical tissue engineered neural scaffolds for CNS and PNS nerve regeneration.

To achieve maximum axonal regeneration and functional recovery in the CNS, it is also necessary to inhibit scar tissue formation after injury, prevent discontinuity during phagocytosis of dying cells, and guarantee adult neuron viability for initial axonal extension (Ellis-Behnke et al., 2006). Researchers have begun to utilize nanotechnology (such as nanomaterials and 3D nanofabrication techniques) to address these obstacles and improve cell proliferation, migration, and differentiation in various biomimetic scaffolds (Ellis-Behnke et al., 2006; Kim et al., 2014; Kumar, Aadil, Ranjan, & Kumar, 2020; Malarkey et al., 2009; Marchesan, Ballerini, & Prato, 2017; Matson & Stupp, 2011; Saito et al., 2009; Wei et al., 2013; Zhang et al., 2005). In particular, biologically inspired nanomaterials exhibiting similar dimensions with tissue ECM can facilitate neural cell growth and guided neural tissue regeneration (Zhang & Webster, 2009). For instance, carbon-based nanomaterials are extensively investigated in neural regeneration due to their outstanding electrical conductivity, excellent mechanical strength, and modifiable surface chemistry (Hu, Ni, Montana, Haddon, & Parpura, 2004). In addition, nanotechnology in tissue engineering readily involves the fabrication of 3D nanostructured scaffolds for improved tissue regeneration (Saracino, Cigognini, Silva, Caprini, & Gelain, 2013). Currently, two of the widely used nanofabrication techniques typically include self-assembling and electrospinning (Cunha et al., 2011). The bottom-up self-assembly process commonly refers to the spontaneous formation of a scaffold from specific nanoscale amphipathic molecules, while electrospinning utilizes biocompatible polymeric materials to fabricate nanofibrous scaffolds. All of these nanostructured scaffolds have illustrated promising results in bridging injured gaps and recovering nerve function (Cao, Liu, & Chew, 2009; Iwasaki et al., 2014; Panseri et al., 2008; Xie, Macewan, Schwartz, & Xia, 2010).

Although conventional 3D nano/microscaffold fabrication approaches have yielded favorable effects in repairing nerve injuries, intrinsic limitations exist with regard to adequate control of scaffold outer shape and internal architecture. To circumvent this problem, 3D bioprinting has garnered greater attention in the production of 3D scaffolds with the exact spatial distribution and microstructural cues (Schmidt & Leach, 2003). It allows for the printing of viable cells, bioactive factors, and biomaterials individually or in tandem, layer-by-layer, resulting in complex tissue substitutes with controlled shape and structure based on predesigned models (Ozbolat & Yu, 2013). Furthermore, the capacity of 3D bioprinting to manufacture patient-specific scaffolds renders it superior to other traditional 3D tissue manufacturing techniques with a rapidly growing interest in the neural tissue engineering field. With the advancement of smart material science, 3D printing has evolved into 4D printing which introduces an additional dimension of time, conferring the 3D-printed products the capacity of shape change upon external stimulation postprinting (Miao et al., 2017; Zhu, Webster, & Zhang, 2019). This chapter provides an overview of the cutting-edge 3D/4D bioprinting and nanotechnology strategies for nerve regeneration. We will focus on (1) recent development of novel nanomaterials, including self-assembling nanomaterials, carbon-based nanomaterials, and conventional biomaterials for nanoneural scaffold design, and (2) 3D/4D bioprinting techniques for the fabrication of customized neural scaffolds.

**FIGURE 16.1**

(A) Schematic illustration of an ideal tissue engineered neural scaffold. (B) Schematic illustration of tissue engineered neural scaffolds for nerve repair. In particular, the left image in (B) represents electrospun random or highly aligned scaffolds for repairing peripheral nerve transection and spinal cord injuries. The right image in (B) represents hydrogel scaffolds for healing spinal cord and traumatic brain injuries.

(B) Adopted from Saracino, G. A. A., Cigognini, D., Silva, D., Caprini, A., & Gelain, F. (2013). *Nanomaterials design and tests for neural tissue engineering*. Chemical Society Reviews, 42, 225–262.

16.2 NANOTECHNOLOGY FOR NEURAL TISSUE REGENERATION

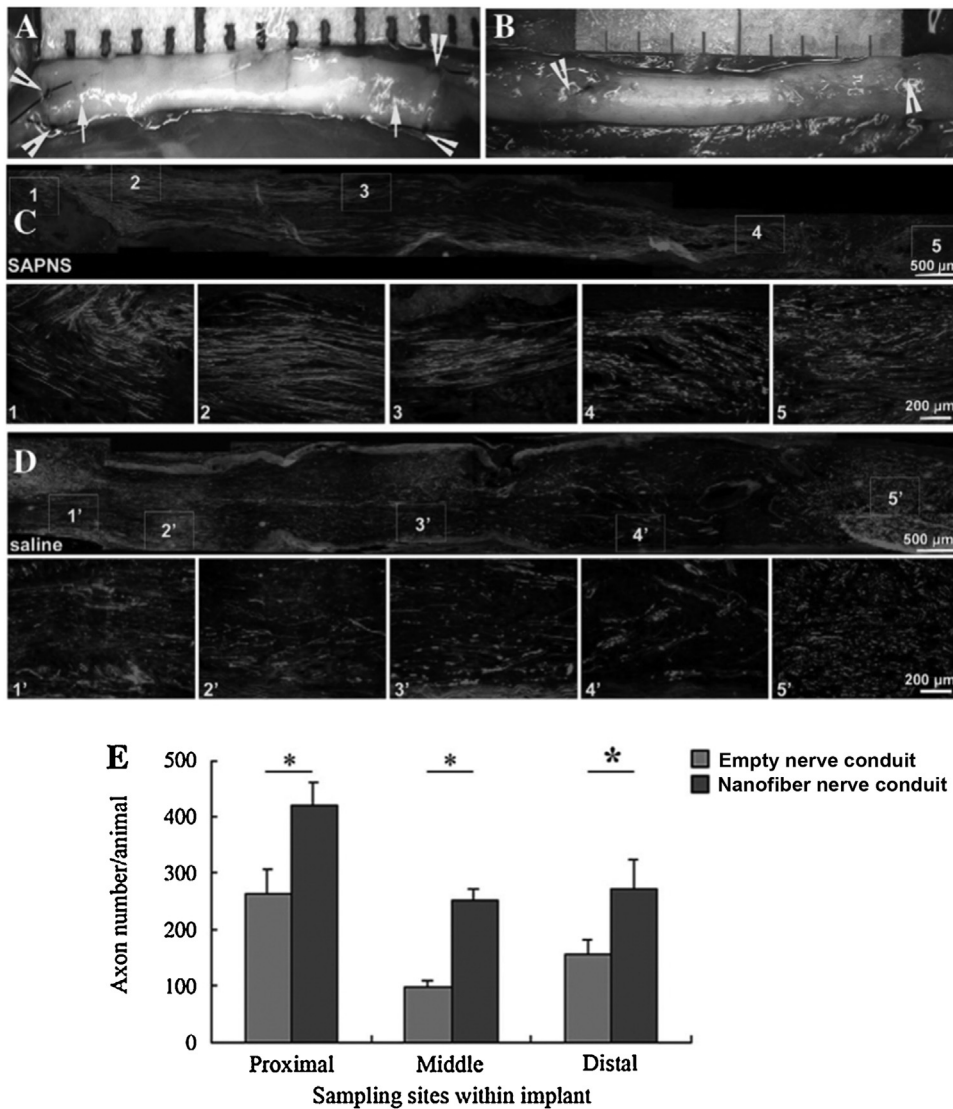
16.2.1 SELF-ASSEMBLING NANOBOMATERIALS

Self-assembly is a process wherein individual biological components (including viruses, peptides, proteins, RNA, and DNA complexes) organize spontaneously into nanostructures, including nanofibers, nanotubes, vesicles, helical ribbons, and β -sheets (Hosseinkhani, Hong, & Yu, 2013; Ko et al., 2010; Li, Xing, Bai, & Yan, 2019; Qiu, Chen, Tang, & Zhao, 2018; Smith, Tejeda-Montes, Poch, & Mata, 2011; Yang, Zhao, et al., 2013; Zhang, Ramsaywack, Fenniri, & Webster, 2008). Self-assembling dynamics can be readily influenced by environmental alterations, such as pH, temperature, ionic strength, presence of specific solutes, and mechanical and electrical stimulation (Xu & Kopeček, 2007). These alterations trigger multiple noncovalent interactions, including hydrogen bonding, electrostatic association, and van der Waals forces, which drive the formation of self-assembling structures (Stephanopoulos, Ortony, & Stupp, 2013). Natural tissue ECM is composed of various nanostructured components that can spontaneously self-assemble. Therefore the use of biomimetic self-assembling nanobiomaterials holds high potential in stimulating cell functions and new tissue formation (Hauser & Zhang, 2010; Zhang & Webster, 2009). In the following, we will discuss several self-assembling peptides used for neural tissue engineering. In addition, other self-assembling nanomaterials such as self-assembly bacteriophage and emerging self-assembling nanotubes will be introduced as well.

Peptide amphiphiles are one of the most popular biological molecules for the fabrication of self-assembling nanobiomaterials. Peptides containing both hydrophilic and hydrophobic moiety have been shown to readily self-assemble into nanofibers, wherein the hydrophilic component forms the outer layer and the hydrophobic component is directed toward the core (Yang et al., 2009). They are much more tractable and scalable when compared to complex biomolecules, such as proteins. Particularly, β -sheet-forming peptides exhibit the unique capability of assembling into one-dimensional (1D) nanostructures via the formation of intermolecular hydrogen bonding (Lock et al., 2013). These 1D nanostructures could be used as building blocks to construct complex 3D networks. In addition, these peptide amphiphiles can be readily modified for enhanced biocompatibility and biodegradability through selecting specific amino acid sequences or controlling over their self-assembling structures (Cui, Webber, & Stupp, 2010; Maude, Ingham, & Aggeli, 2013).

Self-assembling peptide nanofibrous scaffolds (SAPNS) have been used to regenerate PNS and CNS (brain and spinal cord) both in vitro and in vivo. Zhan et al. reported that a novel nanofibrous conduit comprises a blood vessel and filled with RADA16-I (Ac-RADARADARADARADA-CONH₂) SAPNS to repair a 10 mm transection in a rat sciatic nerve (Zhan et al., 2013) (Fig. 16.2). Compared to a blood vessel only control, the SAPNS-incorporated graft enabled peripheral axons to bridge the 10 mm gap as well as significantly enhance motor neuron protection, axonal regeneration, and remyelination. The functional recovery illustrates the great potential of SAPNS-based conduits for the regeneration of peripheral nerve defects.

In another work, Liang et al. successfully accelerated brain tissue regeneration via a novel SAPNS assembled by RADA4 (arginine–alanine–aspartic acid–alanine) peptide placed upon a cortical gray matter lesion (Liang et al., 2011). Combined with noninvasive manganese-enhanced magnetic resonance imaging, the chronic optic tract lesion displayed regeneration of axons leading to near-complete repair and return to function. In addition, Ellis-Behnke et al. used RADA16-I

**FIGURE 16.2**

A sample of blood vessel sheathed self-assembling peptide neural conduit bridging the defected sciatic nerve at the time of (A) immediate after surgery and (B) 16 weeks postimplantation. Arrows indicate the sutured connections. NF200-labeling axons regenerated images in (C) the self-assembling nanofiber filled conduit and (D) empty conduit groups. (E) Axon regeneration comparisons at proximal, middle, and distal part in two neural conduits.

Adopted from Zhan, X., Zhang, W., Guo, J., Wu, W., Gao, M., Jiang, Y., ... Dai, X. (2013). Nanofiber scaffolds facilitate functional regeneration of peripheral nerve injury. *Nanomedicine: Nanotechnology, Biology, and Medicine*, 9, 305–315.

SAPNS to create a permissive environment for brain injury regeneration (Ellis-Behnke et al., 2006). Their experiments showed that peptide scaffolds have the capacity to direct axons to reconnect to target tissues and return the function of vision using a severed optic tract model in hamsters. Guo et al. investigated the capacity of RADA16-I SAPNS to repair SCIs (Guo et al., 2007). In their study, neural progenitor cells and Schwann cells were isolated from rats and implanted into the transected dorsal column of the spinal cord after culturing with SAPNS. Results showed host cells migrated well in SAPNS, blood vessels grew, and the spinal cord lesion was further bridged.

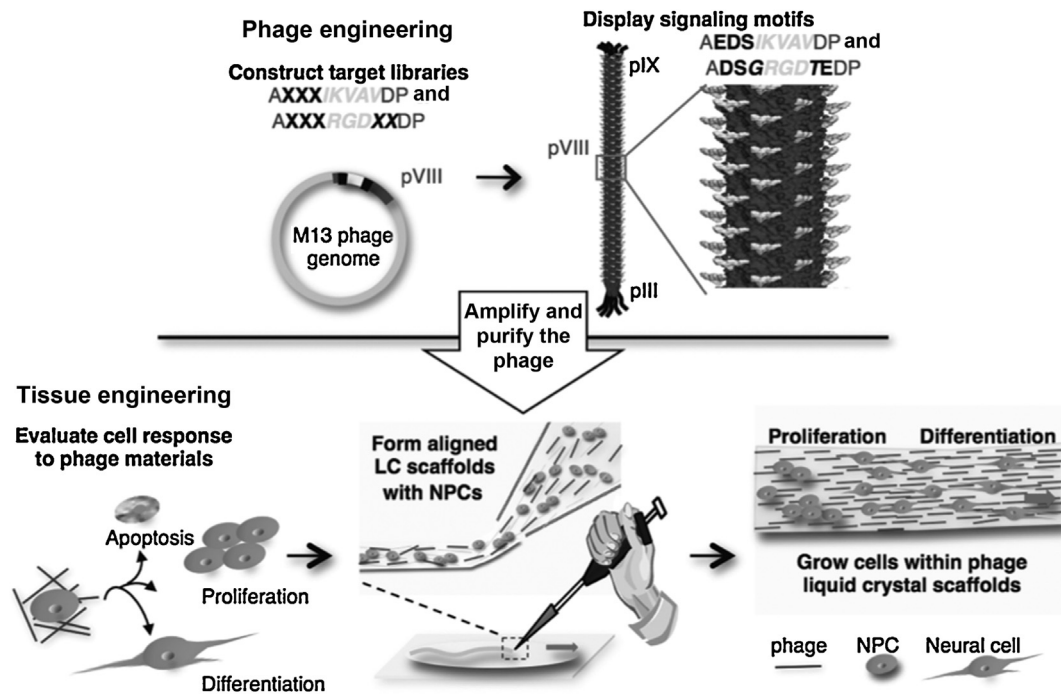
In addition to preassembled SAPNS scaffolds, peptides could be directly injected into injured sites and self-assembled *in vivo*. This specific feature makes them an appealing material for the treatment of SCIs. Once liquid self-assembling peptides are injected into the lesions, they will fill the void, regardless of the shape and size, and assemble into a hydrogel. The direct contact between peptides and ECM requires that self-assembly occurs in a relatively tender environment. In a recent study, Liu et al. showed $K_2(QL)_6K_2$ (QL6) self-assembling scaffolds attenuated posttraumatic inflammation and glial scar formation *in vitro* and *in vivo* (Liu et al., 2013). Unlike most peptides, QL6 is able to self-assemble into β -sheets at neutral pH, which significantly reduced posttraumatic apoptosis, inflammation, and astrogliosis after injection within a clip compression SCI site. *In vitro* experiments revealed that a QL6 scaffold promoted neuronal differentiation, suppressed astrocytic development, increased conduction velocity of axons, reduced refractoriness, and improved high-frequency conduction.

In designing neural scaffolds, controlling the cellular microenvironment is critical for successful tissue regeneration. Self-assembling scaffolds can also be functionalized with various bioactive molecules and binding peptides such as the laminin-derived IKVAV (isoleucine—lysine—valine—alanine—valine) and cell-adherent RGD (arginine—glycine—aspartic acid) moieties for enhanced cellular attachment and neurite outgrowth (Abdul kafi, El-Said, Kim, & Choi, 2012; Cheng, Chen, Chang, Huang, & Wang, 2013; Lu et al., 2018). In the work developed by Tysseling et al., a peptide amphiphile was incorporated on neuroactive epitope IKVAV and injected into mouse SCI (Tysseling et al., 2008, 2010). After 11 weeks, IKVAV PA nanofibers regenerated both descending motor fibers and ascending sensory fibers. They concluded that 3D self-assembling nanofibers with neuroactive epitopes are able to accelerate and improve the regeneration of SCIs. In addition, RDAD-16-I has been functionalized to overcome the drawbacks associated with low pH during self-assembly. Sun et al. developed an SAPNS from two designer self-assembling peptides at neutral pH (Sun et al., 2016). In their study, RGD (cell adhesion peptide) and IKVAV (neurite outgrowth peptide) were separately appended into RADA16-I. These two self-assembling peptides have opposite net charges and can produce nanofibrous hydrogel at neutral pH. This 3D-IKVAV/-RGD SAPNS not only improved cell viability for embedded neural progenitor cells and neural stem cells *in vitro* but also provided a more favorable environment for nerve regeneration when compared to unmodified RDAD-16-I hydrogel *in vivo*. In another study, self-assembling backbone Ac-(RADA)₄-NH₂ was dual-functionalized with IKVAV and a brain-derived neurotrophic factor (BDNF)-mimetic peptide epitope RGIDKRHWNSQ (RGI) in an effort to improve peripheral nerve regeneration (Yang et al., 2020). The dual-functionalized SAPNS promoted rat Schwann cell adhesion, myelination, and neurotrophin secretion *in vitro* and successfully bridged a 10-mm sciatic nerve defect in rats *in vivo*.

Additionally, SAPNS can be used as a slow and sustained functional molecules (bioactive factors, drugs, etc.) release system to protect them from degradation and further enhance tissue regeneration. For instance, Raspa et al. loaded Chondroitinase ABC (ChABC), a drug with

neuroregenerative potential, into SAPNS that resulted in the prolongation of enzymatic activity of ChABC from lasting <72 hours in vitro to 42 days in vitro. Also, injections of this SAPNS loading ChABC favored host neural regeneration and functional recovery in chronic SCI in rats (Raspa, Carminati, Pugliese, Fontana, & Gelain, 2020). In addition, Gelain et al. investigated the diffusive mechanisms of active cytokines within RADA16-I (neutral charge), RADA16-DGE (Ac-RADARADARADARADAGGDGEA-CONH₂) (negative charge), and RADA16-PFS (Ac-RADARADARADARADAGGPFSSTKT-CONH₂) (positive charge) (Gelain, Unsworth, & Zhang, 2010). Results demonstrated that protein mobility is directly related to both physical hindrances and the charge between proteins and peptide nanofibers. Cell studies showed the capability of sustained active cytokine (β FGF) up to 3 weeks when culturing adult neural stem cells. In a study by Bruggeman et al., an SPANS system was generated to reduce growth factor degradation that increased growth factors' lifespan by over 40 times (Bruggeman, Rodriguez, Parish, Williams, & Nisbet, 2016). Specifically, the growth factors were covalently attached to polysaccharide chitosan molecules to increase their interactions with the SPANS. These experiments have opened new avenues for SAPNS-related research with the focus on functional molecular release strategies for neural regeneration.

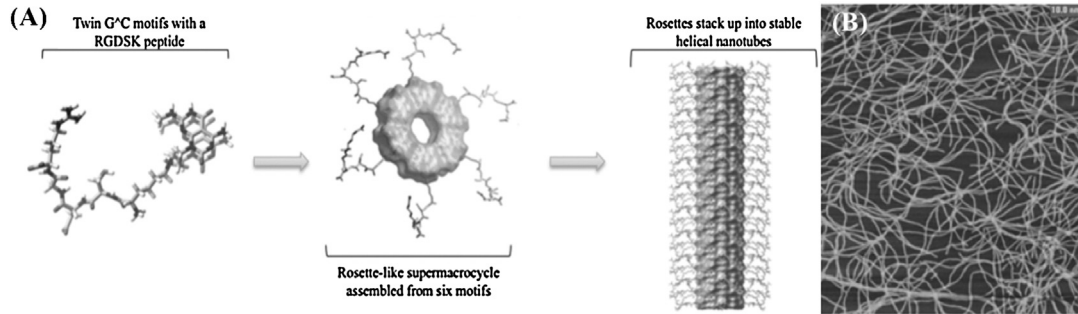
Besides the self-assembling peptide amphiphile nanofibers, several other types of self-assembling nanomaterials have been investigated for neural regeneration. For example, bacteriophage, a human-safe bacteria-specific virus, has been used to generate nanofibrous scaffolds for neural regeneration. Bacteriophage is a monodisperse biological nanostructure, in which the outside surface is made of proteins and nucleic acids are encased in the protein capsid (Cao et al., 2019). It is capable of self-assembling into ordered scaffolds displaying multiple signaling peptide sites. Through controlling the proper patterns or displayed peptides, the generated scaffolds can direct stem cell differentiation into specific cell types (Cao et al., 2019). Merzlyak et al. genetically engineered M13 bacteriophages that naturally form nanofiber-like viral structures displaying signaling motifs on their protein coats for directed self-assembly of nanofibrous scaffolds (Merzlyak, Indrakanti, & Lee, 2009) (Fig. 16.3). After self-assembling into nanofibrous matrices, hippocampal neural progenitor cells were seeded to verify the biological capacity of the novel phage nanobiomaterial. Cell studies exhibited the effectiveness of M13 bacteriophage nanofibrous matrices for enhanced cell viability, proliferation, and differentiation as well as directed 3D cell growth. Another type of self-assembling nanobiomaterial is rosette nanotube (RNT), which has shown substantial promise as a new type of nanobiomaterial for tissue regenerative applications (Fine, Zhang, Fenniri, & Webster, 2009; Sun, Zhang, Hemraz, Fenniri, & Webster, 2012; Zhang, Hemraz, Fenniri, & Webster, 2010; Zhang, Rakotondradany, Myles, Fenniri, & Webster, 2009; Zhang et al., 2008; Zhang, Rodriguez, et al., 2009; Zhou et al., 2020). RNTs are biologically inspired supramolecular nanomaterials formed via the self-assembly of low molecular weight DNA base-pair motifs (Guanine⁺Cytosine, G⁺C, Fig. 16.4). Nanotubular RNTs composed of a hydrophobic core and hydrophilic outer surface remain stable via electrostatic interactions, base stacking, and hydrophobic effects. Morphologically, RNTs exhibit a 3–4 nm outer diameter and several hundred nanometers in length. One critical feature of RNTs is their flexibility in designing their length, diameter, and surface chemistry. Through functionalization of the G⁺C motif, predefined chemical and physical properties for specific tissue regeneration can be achieved. In our lab's work, we explored human bone marrow mesenchymal stem cell (MSC) adhesion, proliferation, and 4 weeks chondrogenic differentiation in twin-based RNTs with cell adherent RGDSK peptide embedded

**FIGURE 16.3**

Schematic illustration of genetically engineered M13 phage and cell response characterization on aligned self-assembling nanofibrous matrices (pVIII, pIII, and pIX represent proteins).

Adopted from Merzlyak, A., Indrakanti, S., & Lee, S.-W. (2009). Genetically engineered nanofiber-like viruses for tissue regenerating materials. *Nano Letters*, 9, 846–852.

within poly-L-lactic acid scaffolds (Childs, Hemraz, Castro, Fenniri, & Zhang, 2013). Our recent study also demonstrated that the lysine-functionalized RNT can promote chondrogenic differentiation of MSCs (Zhou et al., 2020). These results suggested that these biomimetic twin-based nanotubes can significantly enhance MSC growth and chondrogenic differentiation when compared to controls without nanotubes. Both the biomimetic nanostructure and high density of peptides with well-organized architecture contributed to the greatly enhanced stem cell functions in vitro. Theoretically, any cell-favorable short peptides can be conjugated onto the G⁺C motifs to modulate surface chemistry, rendering RNTs as a biomimetic nano-template for a variety of tissue/organ regeneration. For instance, neurogenic peptides can be easily conjugated onto RNTs with controlled spatial distribution and density to improve specific protein adsorption as well as cell function during neural regeneration. In a study by Puzan et al., it was found RGDSK-functionalized RNT blends supported dorsal root ganglion neuron attachment, and neurite extension and sprouting (Puzan, Legesse, Koppes, Fenniri, & Koppes, 2018). The RGDSK-functionalized RNT presented increasing neurite outgrowth by up to 47% over a 7-day period compared with RNT with a lysine side chain.

**FIGURE 16.4**

Schematic illustration of the self-assembly process of RNTs. (A) Six twin G^C motifs with a RGDSK peptide are self-assembled into rosette-like supermacrocycles and then stack up into stable helical nanotubes. RNTs with a 11 Å hollow core are 3–4 nm in diameter and up to several μm long. (B) Atomic force microscopy image of the RNTs with RGDSK.

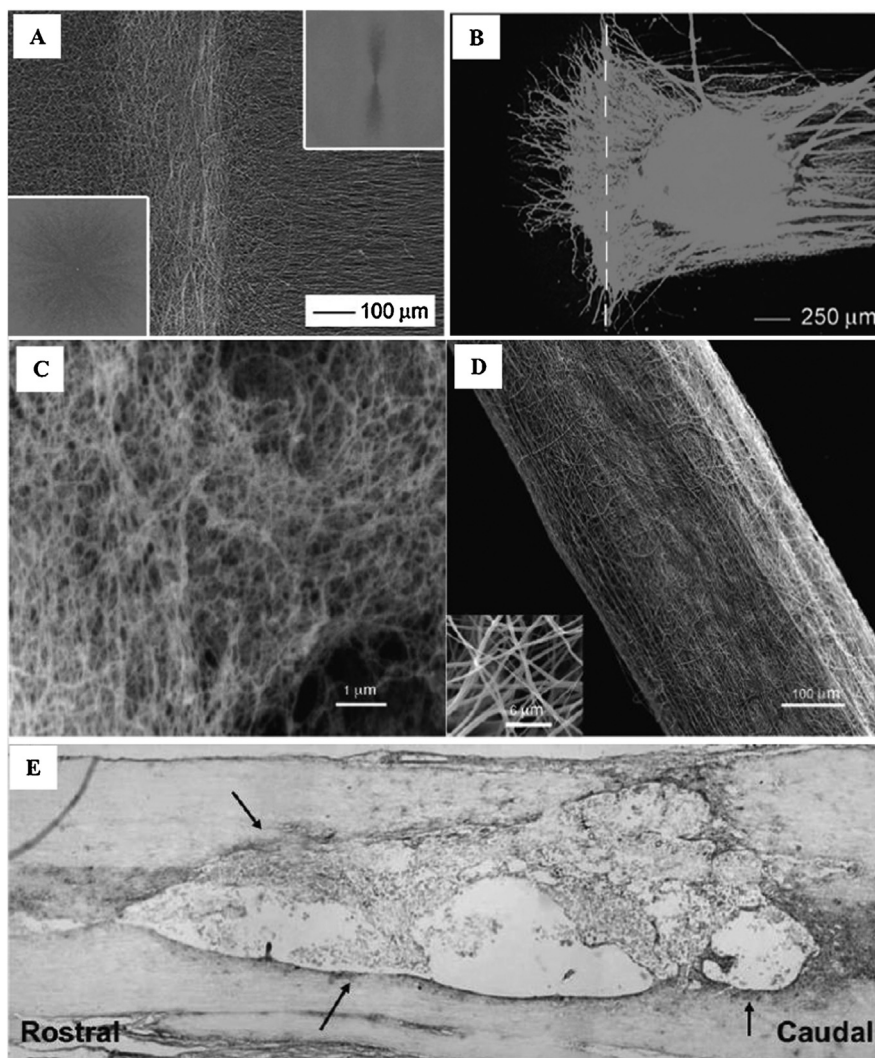
16.2.2 ELECTROSPUN POLYMERIC NANOFIBROUS NEURAL SCAFFOLD

To repair a complex neural network, guided neurite outgrowth is required toward the synaptic targets with spatially distributed cell populations placed into a specific pattern (Xie, Macewan, Li, Sakiyama-Elbert, & Xia, 2009). Aligned nanofibrous scaffolds can provide patterned cues to achieve this goal. Over the past decade, the development of innovative nanofibrous scaffolds has greatly enhanced the involvement of polymers in neural regeneration. Among various nanofibrous scaffold fabrication techniques, electrospinning has been widely investigated for neural tissue regeneration due to its versatility in implementing a variety of synthetic and natural polymeric biomaterials for the creation of highly aligned fibrous scaffolds. These scaffolds have shown promising results due to their structural similarity to fibrous proteins found in native ECM thus facilitating the outgrowth of neurites (Dahlin, Kasper, & Mikos, 2011; Ma, Kotaki, Inai, & Ramakrishna, 2005; Sabra, Ragab, Agwa, & Rohani, 2020).

Electrospinning could be easily tailored by altering polymeric solutions and processing parameters to fabricate scaffolds with varying mechanical and biological properties, as well as fiber and gross scaffold morphology (Sill & von Recum, 2008; Theron, Zussman, & Yarin, 2004; Vasita & Katti, 2006). More than 100 types of natural and synthetic polymers have been employed in electrospinning for various tissue engineering applications (Burger, Hsiao, & Chu, 2006). Biodegradable polyesters, including polycaprolactone (PCL), poly(lactic-co-glycolic acid) (PLGA), poly(lactic acid) (PLA), etc., are the most popular synthetic polymers currently used for neural tissue engineering applications. These polymers can be easily tailored for specific mechanical properties and topography by altering the fiber diameter, which can range from nano- to microscale for improved cell response (Keun Kwon, Kidoaki, & Matsuda, 2005; Kumbar, James, Nukavarapu, & Laurencin, 2008). Studies have demonstrated the effects of fiber diameter on neural stem cell proliferation and differentiation. Christopherson et al. investigated hippocampus-derived adult neural stem cell response to laminin-coated electrospun polyethersulfone meshes with various fiber diameters (283 ± 45 nm, 749 ± 153 nm, and 1452 ± 312 nm) (Christopherson, Song, & Mao, 2009). They found cells exhibited a

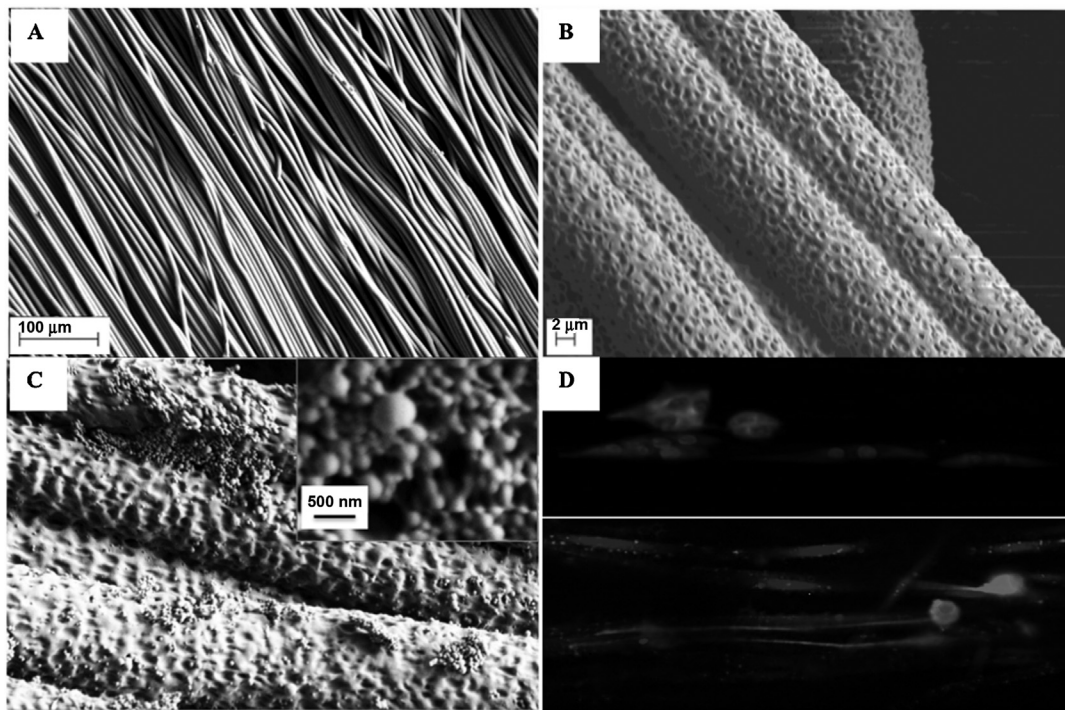
trend of improved proliferation and spreading, and reduced aggregation with decreasing fiber diameter. In addition to fiber diameter, another topographical cue, fiber orientation, has been shown to play a critical role in neural regeneration. Oriented fibers can serve to guide neurite extension to target injured tissues or organs. Xie et al. examined the neurite outgrowth of primary dorsal root ganglia on electrospun PCL nanofibers with varied fiber orientation, structure, and surface properties (Xie et al., 2009). They found neurites illustrated radial distribution when cultured on randomly oriented nanofibers. A parallel array of aligned nanofibers, by contrast, guided neurite extension along the direction of the nanofiber. Interestingly, when cultured at the border between aligned and random nanofibers, the same dorsal root ganglia exhibited neurite outgrowth in response to the underlying aligned and randomly oriented nanofibers, respectively (Fig. 16.5A and B). To achieve further improved cytocompatibility, synthetic polymers can be coelectrospun with natural polymers such as chitosan, fibrin, and collagen or integrated with self-assembling nanobiomaterials which mimic natural ECM chemistry. Sadeghi et al. fabricated nanofibrous scaffolds by coelectrospinning PCL, polypyrrole (PPy), and chitosan to combine advantages of nanofiber's topography with advantages of chitosan and PPy (Sadeghi, Moztaaradeh, & Aghazadeh Mohandesi, 2019). The average diameter of the electrospun fibers ranged from 30 to 180 nm, which was influenced by the concentration of the chitosan. Increasing 30% in chitosan content decreased fiber diameter from 124 to 36 nm. Meanwhile, chitosan imposed significant advancement in improving the hydrophilicity of the electrospun scaffold. PC12 cells growing on this PCL/chitosan/PPy nanofibrous scaffold presented enhanced cell attachment, spreading, as well as increased cell proliferation up to 356% in comparison to that on pristine PCL. In a study by Gelain et al., a novel neural guidance channel was fabricated by combining electrospun nanofibers and self-assembling peptides (Fig. 16.5C–E) (Gelain et al., 2011). Self-assembling peptide RADA16-I-BMHP1 (Ac-RADARADARADARADAGGPFSSSTKT-CONH₂) was assembled into PLGA/PCL blended electrospun microchannels followed by implantation within a chronic SCI rat model. New tissue formation was observed and conspicuous cord reconstruction accompanied the improvement of ascending and descending motor pathways and global locomotion score after 6 months. Besides these modification methods, our laboratory has tried carbonized electrospun scaffolds to reduce the size of fibers and increase electrical conductivity (Zhu, Ye, et al., 2018). The electrospun scaffolds were firstly prepared with the fiber size of 780 nm followed by carbonization with elevated temperature. This carbonized process reduced the average size of fibers to 384 nm, as well as rendered the electrical conductivity to scaffolds. When the neural stem cells grown on the carbonized scaffold were stimulated by electricity, cell proliferation, neuronal differentiation, and maturation were all promoted.

In addition to chemical and morphological modifications, researchers have begun to incorporate growth factors within the scaffold to further enhance neural tissue regeneration. A study conducted in our laboratory investigated the effects of a highly aligned fibrous neural scaffold using electrospinning in conjunction with electrosprayed growth factor encapsulated nanospheres (Fig. 16.6). Results demonstrated that aligned scaffolds with sustained growth factor release can improve PC-12 cell growth and effectively guide neural axon extension along with the orientation of fiber(s). In a study by Hu et al., PCL-nerve growth factor (NGF) and bovine serum albumin (BSA) nanofibrous scaffolds were fabricated by emulsion electrospinning technique, in which PCL serving as a shell encapsulated NGF and BSA in the core (Hu, Tian, Prabhakaran, Ding, & Ramakrishna, 2016). BSA can stabilize the NGF from emulsion electrospun nanofibers and keep its sustained release for over 28 days.

**FIGURE 16.5**

(A) SEM image of fiber mat with connected random and aligned fiber sand. (B) Dorsal root ganglia cell growth on the border of random and aligned nanofibers. The white dashed line represents the borderline between aligned (right side) and randomly oriented (left side) fibers. The neural conduit fabricated by integrating (C) self-assembled RADA16-I-BMHP1 and (D) PLGA/PCL electrospun channel, and (E) Hematoxylin-esion staining of injured spinal cord after implantation, arrows point the cyst margins.

(A, B) Adopted from Xie, J., Macewan, M. R., Li, X., Sakiyama-Elbert, S. E., & Xia, Y. (2009). Neurite outgrowth on nanofiber scaffolds with different orders, structures, and surface properties. *ACS Nano*, 3, 1151–1159; (C–E) Adopted from Gelain, F., Baldissera, F., Vescovi, A., Panseri, S., Antonini, S., Cunha, C., ... Montagna, M. (2011). Transplantation of nanostructured composite scaffolds results in the regeneration of chronically injured spinal cords. *ACS Nano*, 5, 227–236.

**FIGURE 16.6**

SEM images of (A) an aligned electrospun neural scaffold fabricated in our laboratory; (B) random nanopores created on the surface of the electrospun fibers via solvent evaporation; and (C) electrospayed core-shell nanospheres incorporated aligned scaffold. (D) Confocal images of axons extension along the direction of aligned fibers (red represents the cell skeleton and axons).

Another intriguing feature that can improve neural generation is a scaffold's electrical conductivity. Studies have shown that electrical stimulation can also improve the communication between nerve and muscle cells, and further promote neural network formation and functional recovery (Ghasemi-Mobarakeh, Prabhakaran, Morshed, Nasr-Esfahani, & Ramakrishna, 2009; Huang et al., 2012; Zhang, Xin, Tong, & Tong, 2013). With regard to this aspect, electrically conductive polymers have offered an attractive option in neural tissue engineering (Chronakis, Grapenson, & Jakob, 2006). Two approaches currently employed in the fabrication of conductive electrospun nanofibrous scaffolds rely on the use of inherently electrically conductive polymeric biomaterials such as polypyrrole (PPy) and polyaniline (PANI): either directly dope conductive polymer into electrospun solution or coat nonconductive fibers with a conductive polymer (Ghasemi-Mobarakeh et al., 2009; Jin et al., 2012; Lee, Bashur, Goldstein, & Schmidt, 2009; Zamani, Amani-Tehran, Zaminy, & Shokrgozar, 2017). PPy is one of the most widely studied neural biomaterials due to its ease of synthesis, excellent cytocompatibility, and excellent conductivity (Lee et al., 2009). Both in vitro and in vivo studies have shown that PPy-doped or -coated neural substitutes are suitable for implantation (Brett Runge et al., 2010; George et al.,

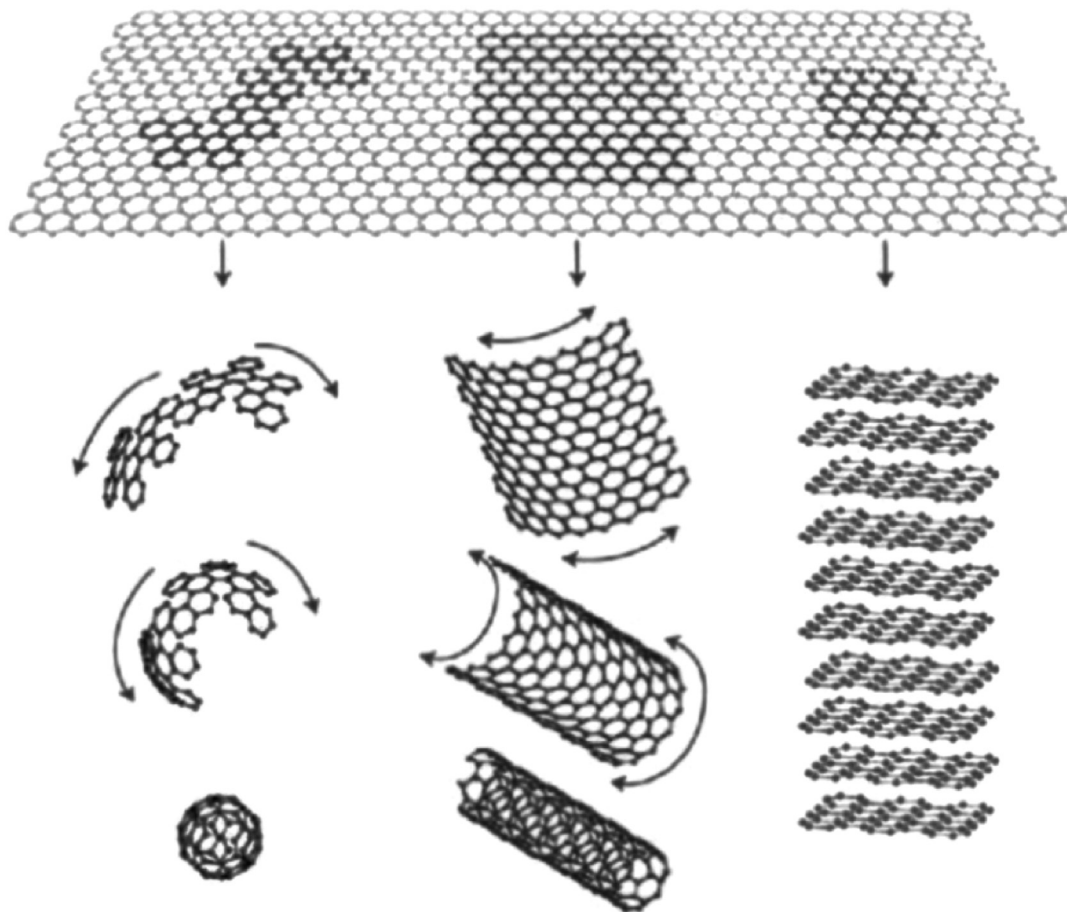
2005; Xu et al., 2014). It was also revealed that nanofibrous scaffolds fabricated through electrospinning PCL/gelatin doped with conductive PANI could significantly improve nerve stem cell proliferation and neurite outgrowth under electrical stimulation when compared with the absence of electrical stimulation group (Ghasemi-Mobarakeh et al., 2009).

Besides electrospinning, touch-spinning has been developed to produce nanofibrous scaffolds for tissue engineering applications. Touch-spinning produces nanofibers through mechanical control in the absence of electricity (Tokarev et al., 2015). When compared to electrospun nanofibers, touch-spun nanofibers undergo a greater chain stretching and alignment, leading to an increased crystallinity (Asheghali et al., 2020; Lee et al., 2020). The touch-spun nanofibrous scaffolds presented improved stability in the aqueous cell culture environment which prevented nanofibers from misalignment and entanglement even if the scaffolds were swelled for 2 weeks and then underwent a 14-day neural differentiation (Asheghali et al., 2020; Lee et al., 2020). These results suggested that the neurites on the touch-spun nanofibers can be directed to a preferred orientation even after long-term swelling.

16.2.3 CARBON NANOBIMATERIALS

The unique and versatile properties of carbon nanobiomaterials, including excellent electrical, thermal, and mechanical properties, have led to greater research interest. A variety of carbon nanotubes/nanofibers (CNTs/CNFs) and graphene nanomaterials (Bei et al., 2019; Lee et al., 2018; Reddy et al., 2018; Tran, Zhang, & Webster, 2009; Wang, Wang, Li, Li, & Lin, 2011) have attracted considerable attention for use as novel conductive scaffold materials for neural tissue regeneration.

Graphene is a carbon sheet measuring one atom in thickness composed of sp^2 -bonded carbon atoms condensed in a hexagonal 2D lattice (Fuhrer, Lau, & Macdonald, 2010; Katsnelson, 2007). Due to this unique structure, it is the thinnest and strongest material known (Geim, 2009). As a basic building block of all graphitic forms, graphene can be wrapped into 0D fullerenes, rolled in to 1D CNTs, and stacked into 3D graphite (Geim & Novoselov, 2007; Rao, Sood, Subrahmanyam, & Govindaraj, 2009) (Fig. 16.7). The unique physical and chemical properties of graphene and graphene-based nanomaterials, such as high planar surface area, electronic flexibility, superlative mechanical strength, and unparalleled thermal conductivity, have increased their use in biological applications (Reddy, He, Ramakrishana, & Luo, 2019; Yang, Asiri, Tang, Du, & Lin, 2013). Graphene, graphene oxide, chemically modified graphene, and other graphene derivatives are some of the more popular candidates for biomedical applications (Wang et al., 2011). With regard to biocompatibility, Park et al. studied nerve cell growth on the surface of graphene showing comparable cell viability when compared to a polystyrene surface (Park, Nam, et al., 2013). Furthermore, Tang et al. investigated the formation and performance of neural networks on graphene films (Tang et al., 2013). It was demonstrated that graphene has the capacity to improve nerve cell performance, promote the growth, and facilitate electrical signaling of neural circuits. Such studies revealed the potential of graphene as an excellent candidate for neural tissue engineering. In addition to 2D graphene films, a study by Li et al. investigated neural stem cell growth on 3D graphene foams (Li et al., 2013). It was found that neural stem cells can actively proliferate on 3D graphene when compared to 2D graphene films. In addition, 3D graphene promoted neural stem cell differentiation to astrocytes and neurons, as well as efficiently mediated electrical stimulation for differentiated

**FIGURE 16.7**

Graphene forms: fullerenes, carbon nanotubes, and graphite.

Adopted from Geim, A. K., & Novoselov, K. S. (2007). The rise of graphene. Nature Materials, 6, 183–191.

neural stem cells. Graphene is usually supplemented into polymeric scaffolds to improve neural tissue regeneration. In a study by Niu et al., graphene was blended into silk fibroin to obtain conductive scaffolds, which led to enhanced neuronal differentiation of induced pluripotent stem cells grown on scaffolds (Niu et al., 2018). When the electrical stimulation was applied, graphene-containing scaffolds also showed improved neural cell response compared to pristine polymeric scaffolds. In Wang et al.'s study, they also demonstrated that electrical stimulation elevated differentiation of PC12 grown on graphene-oxide-coated scaffolds (Wang et al., 2019). The in vivo implantation of their conductive scaffolds was studied using a rat sciatic nerve defect model. Results illustrated their conductive scaffolds coated with grapheme oxide exhibited similar healing capacities to autografts.

CNTs are secondary structures composed of single- or multilayered graphene sheets, which have been rolled into a tubular structure resulting in the formation of single-walled carbon nanotubes (SWCNTs) or multiwalled carbon nanotubes (MWCNTs) (Dai, 2002). The incorporation of CNTs within nanocomposite scaffolds can create neural interfaces with the increased interfacial area, conductivity, and electrochemical stability (Keefer, Botterman, Romero, Rossi, & Gross, 2008). Both *in vitro* and *in vivo* experiments have been conducted to investigate the cytotoxicity and biocompatibility of CNTs for potential use in neural regenerative applications. For instance, Aldinucci et al. studied the immune-modulatory action of human dendritic cells on MWCNTs *in vitro* (Aldinucci et al., 2013). Based on their findings, differentiated and activated dendritic cells presented a lower immunogenic profile when interfaced with MWCNTs and the immune reaction modulation was related to topographical and physical features of the growth surface. It was also found that neuronal viability of postnatal mouse dorsal root ganglia was reduced when exposed in a higher concentration of MWCNTs containing culture media (Gladwin, Whitby, Mikhlovsky, Tomlins, & Adu, 2013). In that study, 250 $\mu\text{g/mL}$ of MWCNT-containing media exhibited neuronal death and abnormal neurite morphology while 5 $\mu\text{g/mL}$ of MWCNT-containing media presented no cytotoxicity over 14-day culture. Although there have been cytotoxicity concerns raised about CNTs; thus far, the exact mechanisms of CNT's effects on cells are still not fully known. But the results show that nanotubes are not cytocompatible only under certain conditions, for example, certain tube lengths, high concentration, hydrophobicity of bare nanomaterial, and dispersion of nanotubes. It was suggested that CNTs can serve as an excellent nanobiomaterial for neural regeneration through the surface and structural modifications for enhanced biocompatibility and cell growth. In the following, we will briefly overview the research progress in this field.

As we know, the success of tissue regeneration depends heavily on initial cell–scaffold interaction, which is largely determined by the surface properties of the scaffold (Vasita, Shanmugam, & Katti, 2008). Modification of surface charge/chemistry as well as the integration of signaling complexes on the CNT surfaces can regulate cell adhesion, proliferation, differentiation, matrix remodeling, and tissue organization (Yang, Thordarson, Gooding, Ringer, & Braet, 2007). To date, multiple strategies including topographical alterations and chemical functionalization have been explored to modify CNT-based scaffold surface. With regard to topographical alteration, Fan et al. designed superaligned CNT yarn scaffolds and demonstrated neurite outgrowth extended along with the CNT yarns with decreased branching (Fan et al., 2012). Similarly, Bédurier et al. investigated neuron growth on SiO_2 substrates patterned with double-walled CNTs (Bédurier et al., 2012). It was found that neurons were able to sense the physical and chemical properties of the scaffold surface in a contact-dependent manner. Furthermore, neurons prefer to grow on substrates with patterned CNTs, and directed neurite outgrowth can also be modulated by micrometric CNT patterning. It is postulated that directed neuronal outgrowth is attributed to the preferential adsorption of specific culture medium proteins. In addition, the topography of CNTs impacts the neural differentiation of stem cells when exposed to a chemical inducer. In a study by Park et al., MSCs cultured in neural differentiation media showed enhanced neural gene expression when grown on linear CNT networks when compared to bulk randomly oriented CNT-based films (Park, Kang, & Hong, 2013). In another study, Park et al. developed a CNT network pattern for selective growth and controlled neuronal differentiation of human neural stem cells into neurons (Park et al., 2011). They illustrated that the patterned CNT network could provide synergistic cues by selective laminin adsorption and optimal nanotopography guidance. The synergistic cues successfully controlled the neuronal differentiation at the level of individual axon or neurite.

In addition to topographical modification, CNTs can be readily functionalized through various chemical methods to sites at the end, side, or induced defect sites (Peng & Wong, 2009). Treatment with strong acids (such as HNO_3 or H_2SO_4) or other oxidative agents will generate oxygenated groups (such as carboxylic acid groups) at CNT ends or defect sites, which can undergo further modification by linking specific functional groups (Banerjee, Hemraj-Benny, & Wong, 2005; Bekyarova et al., 2005). Chemical functionalization can alter the physical and chemical properties of CNTs including solubility, electrical, and mechanical properties (Balasubramanian & Burghard, 2005; Bekyarova et al., 2005; Hirsch, 2002; Matsumoto, Sato, Naka, Whitby, & Shimizu, 2010; Ni et al., 2005). Several studies have shown the potential of functionalized CNTs to improve biocompatibility and cellular responses. Hu et al. demonstrated, for the first time, the influence of functionalized CNTs with various surface charges on the outgrowth and branching pattern of neurons (Hu et al., 2004). Their studies focused on three kinds of functionalized MWCNTs fabricated by covalently conjugating $-\text{COOH}$, $-\text{PABS}$ (poly-*m*-aminobenzene sulfonic acid), and $-\text{EN}$ (ethylenediamine) with negative, neutral, and positive charge at physiological pH, respectively. Neurons grown on MWCNTs with positive surface charge exhibited outstanding cellular response, much more growth cones, extended neurite length, and elaborate neurite branching when compared to neurons grown upon neutral or negatively charged MWCNTs (Fig. 16.8). Based on these findings, numerous functionalized CNTs have now been reported. Gottipati et al. chemically functionalized SWCNT films to modulate the morphology and proliferation of astrocytes (Gottipati et al., 2013). In this work, SWCNTs were covalently linked to polyethylene glycol (PEG) followed by spraying into films of various thicknesses. When compared to astrocytes grown on unmodified samples, cells grown on SWCNT-PEG films were bigger and rounder in morphology, exhibited decreased immunoreactivity of glial fibrillary acidic protein, and promoted cell proliferation that is associated with the dedifferentiation of astrocytes. In a report by Jan and Kotov, SWCNTs were functionalized with polyelectrolyte (PEI) and assembled layer-by-layer to form films (Jan & Kotov, 2007). The SWCNT-PEI induced mouse embryonic neural stem cell differentiation into neurons, astrocytes, and oligodendrocytes with clear neuritis while also exhibiting comparable biocompatibility and expression of neural markers with cells cultured on poly-L-ornithine, one of the most popular substrates for neural stem cells. In addition to single use of CNT or graphene, some studies have tried to use both of them in one scaffold. Liu et al. introduced cross-linkable bonds to CNT and GO to obtain the functionalized CNT poly(ethylene glycol) acrylate and graphene oxide acrylate (Liu et al., 2017). The functionalized CNT and GO were subsequently incorporated into oligo(poly(ethylene glycol) fumarate) (OPF) hydrogel through chemical cross-linking and in situ reductions. The

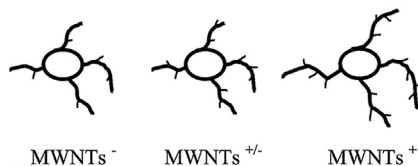


FIGURE 16.8

Schematic of neuron growth on positive-, neutral-, and negative-charged MWCNTs.

Adopted from Hu, H., Ni, Y., Montana, V., Haddon, R. C., & Parpura, V. (2004). Chemically functionalized carbon nanotubes as substrates for neuronal growth. Nano Letters, 4, 507–511.

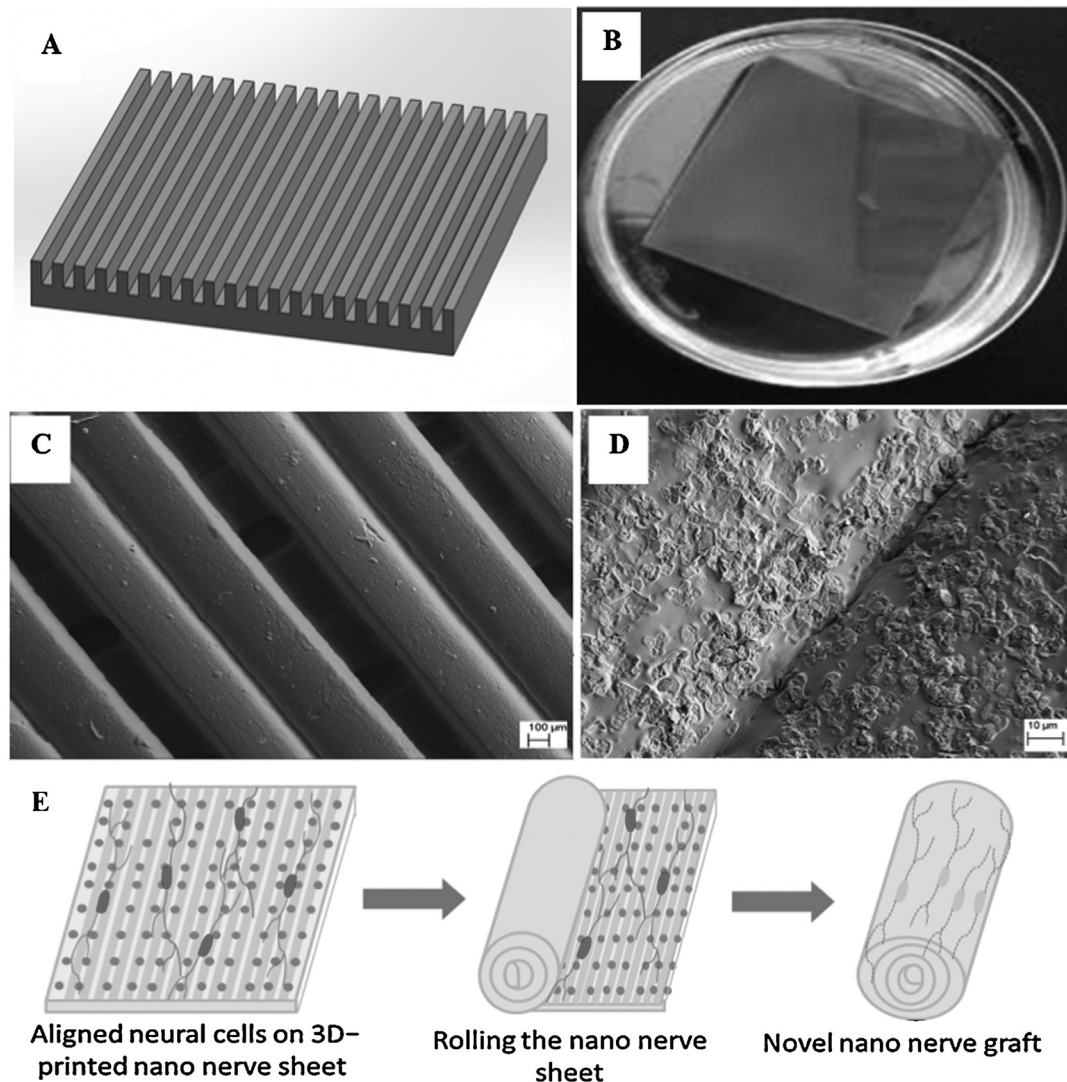
resultant scaffolds were positively charged by 2-(methacryloyloxy)ethyltrimethylammonium chloride (MTAC). The functionalized CNT and GO in the scaffolds presented excellent biocompatibility and enhanced PC12 cell proliferation and spreading, as well as robust neurite development.

16.3 3D/4D BIOPRINTING FOR NEURAL TISSUE REGENERATION

16.3.1 3D BIOPRINTING FOR NEURAL TISSUE REGENERATION

3D bioprinting is achieving a great popularity in tissue engineering as a means of fabricating customized 3D cellular tissue constructs. The outstanding advantage of 3D bioprinting techniques is their capacity to directly produce complex tissue scaffolds with precise spatial distribution and biomimetic architecture. These techniques can be classified into two categories: scaffold printing and cell or cell-containing material printing.

Fused deposition modeling (FDM), stereolithography, and inkjet bioprinting are the popular 3D bioprinting techniques for the manufacture of neural scaffolds. Shepherd et al. fabricated 3D poly(2-hydroxyethyl methacrylate) (pHEMA) neural scaffolds via photolithography (Shepherd et al., 2011). In this study, a photopolymerizable hydrogel ink composed of branched pHEMA chains, HEMA monomer, comonomer, photoinitiator, and water was prepared. The ink was deposited and crosslinked under UV radiation to form a 3D interpenetrating hydrogel network for primary rat hippocampal neuron growth. Results showed that scaffold architecture can be controlled precisely and the structure influenced both cell distribution and aligned extension of neurons. In addition, Melissinaki et al. (2011) explored a photocurable biodegradable PLA-based resin and fabricated scaffolds via a direct laser writing method (Melissinaki et al., 2011). They conjugated photocurable methacrylate groups to PLA resin and crosslinked using a femtosecond Ti:sapphire laser. Resultant porous scaffolds displayed a maximum resolution of 800 nm and enabled guided neuronal growth. In our laboratory, we have developed a novel 3D-printed nano nerve scaffold through the integration of conductive graphene nanobiomaterials with 3D stereolithography (Fig. 16.9). Our results have shown that the construct with graphene nanoplatelets has very good cytocompatibility properties. In addition, the graphene nanoplatelets can greatly improve the conductivity of the scaffold, which make the conductive scaffold promising for neural regeneration. Inkjet printing is another convenient technique to create patterned polymeric structures for the promotion of desired cellular behavior. This technique can readily deposit cell adhesive biomaterials in a precise pattern to guide neural cell growth. A study by Sanjana et al. revealed inkjet-printed collagen/poly-D-lysine (PDL) on a poly(ethylene) glycol surface can support rat hippocampal neurons and glial growth in defined patterns when compared to collagen/PDL absent regions (Sanjana & Fuller, 2004). With the advancement of molecular biology and the development of novel cell favorable factors, biomimetic nanomaterials could be printed on a traditional scaffolds' surface to obtain more cell-favorable features. Moreover, 3D bioprinting has also provided a means for the incorporation of electrically conductive materials within neural scaffolds. Weng et al. inkjet printed PPy/collagen scaffolds and incorporated electrical stimulation into the system (Weng, Liu, Shepherd, & Wallace, 2012). In this study, PPy and collagen were microstructured on polyarylate film by inkjet printing for electrical stimulation of a spatially controlled system. The PPy/collagen track was illustrated to guide PC-12 adherence and growth, while electrical stimulation showed the ability to promote neurite outgrowth

**FIGURE 16.9**

3D-printed aligned PEG-DA neural construct sheet with highly conductive graphene nanoplatelets: (A) is a 3D CAD model of aligned neural construct sheet; (B) photo image of 3D-printed neural construct with graphene nanoplatelets; (C and D) SEM images of the 3D-printed scaffold with grapheme nanoplatelets at low and high magnifications; and (E) schematic illustration of the 3D nerve scaffold in implantation configuration.

and orientation. In one of our studies, we introduced a poly(3,4-ethylenedioxythiophene) (PEDOT)/ polystyrene sulfonate (PSS) aqueous solution to a polyethylene glycol diacrylate (PEGDA) crosslinkable bioink and 3D printed the bioink to achieve conductive 3D-printed scaffolds (Heo et al.,

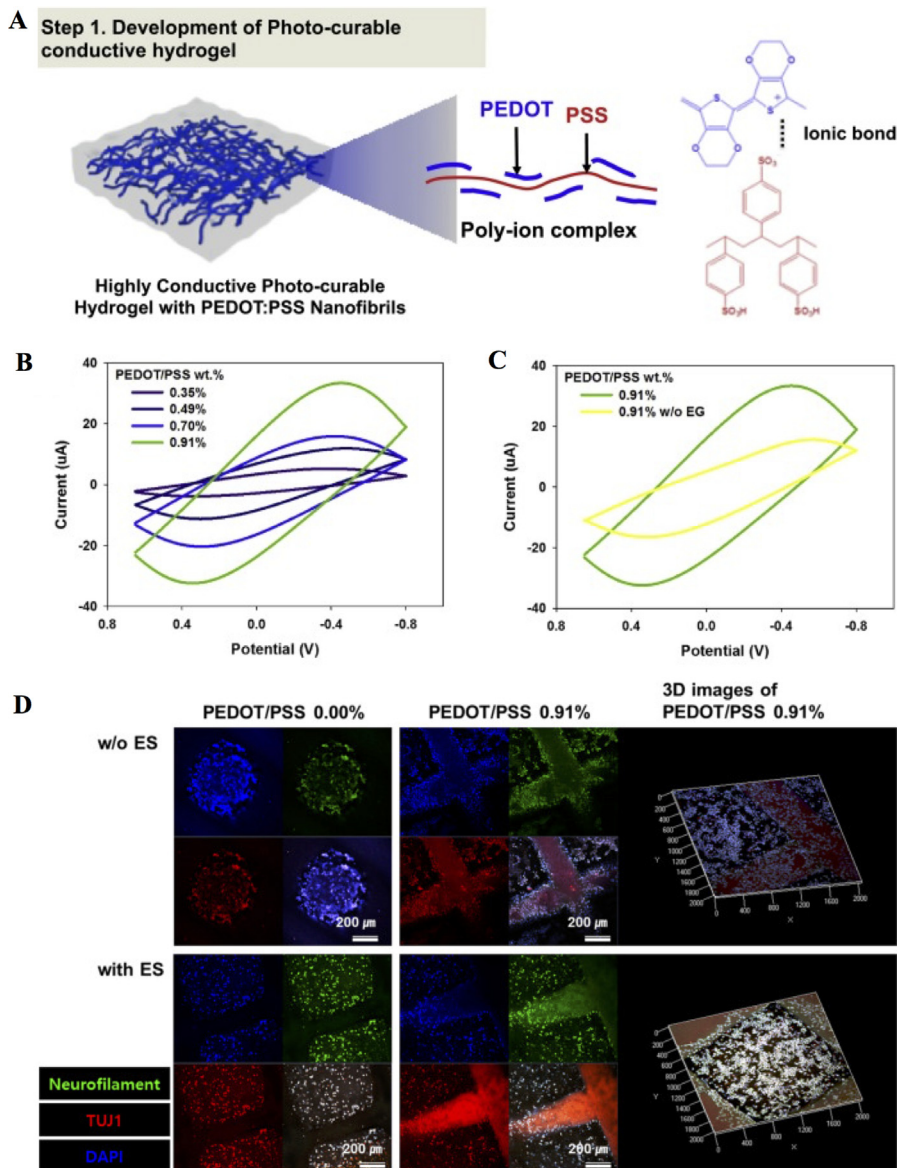
2019). It was found that the resultant scaffolds had significantly increased electrochemical properties with the increase of PEDOT/PSS concentration in the bioink. The electrical stimulation imposed on the scaffolds significantly enhanced neuronal differentiation of dorsal root ganglion (DRG) grown in this system (Fig. 16.10).

3D-printed scaffolds can integrate with other strategies that are beneficial to the rehabilitation of degenerative nerves and neural disorders to achieve a combined or synergistic regenerative effect. In comparison to using a scaffold alone, this comprehensive repair-strategy may provide extra complexity (stimulus) for enhanced neural functional recovery. In one of our studies, we investigated the contribution of low-level light therapy (LLLT) to cells grown on 3D-printed scaffolds (Zhu, George, Sorger, & Grace Zhang, 2017). LLLT's application in biomedicine refers to the use of exposing cells/tissues to light in the wavelength of 300–10,600 nm with a power output of 0.001–0.1 W, pulse rate from 0 to 5000 Hz (Posten et al., 2005). We deployed red laser light stimulation to neural stem cells on 3D-printed scaffolds, and found cell proliferation rate as well as intracellular reactive oxygen species synthesis were greatly increased. Meanwhile, neuronal differentiation of neural stem cells was promoted by the laser stimulation, while the glial differentiation was suppressed. These results suggested that integration of 3D printing and LLLT or other techniques favorable to neural regeneration might provide a powerful strategy for neural tissue engineering.

In addition to the aforementioned 3D scaffold printing technologies that cells were seeded post-printing, there is also great interest in 3D bioprinting neural cells and other native cells in the precise spatial distribution for neural applications. Fig. 16.11 shows several important 3D cell bioprinting systems, including laser-based writing, inkjet bioprinting, and extrusion-based deposition for 3D tissue and organ fabrication (Ozbolat & Yu, 2013). These techniques provide great precision over the spatial placement of cells within the internal architecture of fabricated scaffolds (Melchels et al., 2012). Multiple cell types can be printed collectively thus producing a highly biomimetic ECM microenvironment for facilitated neural tissue regeneration.

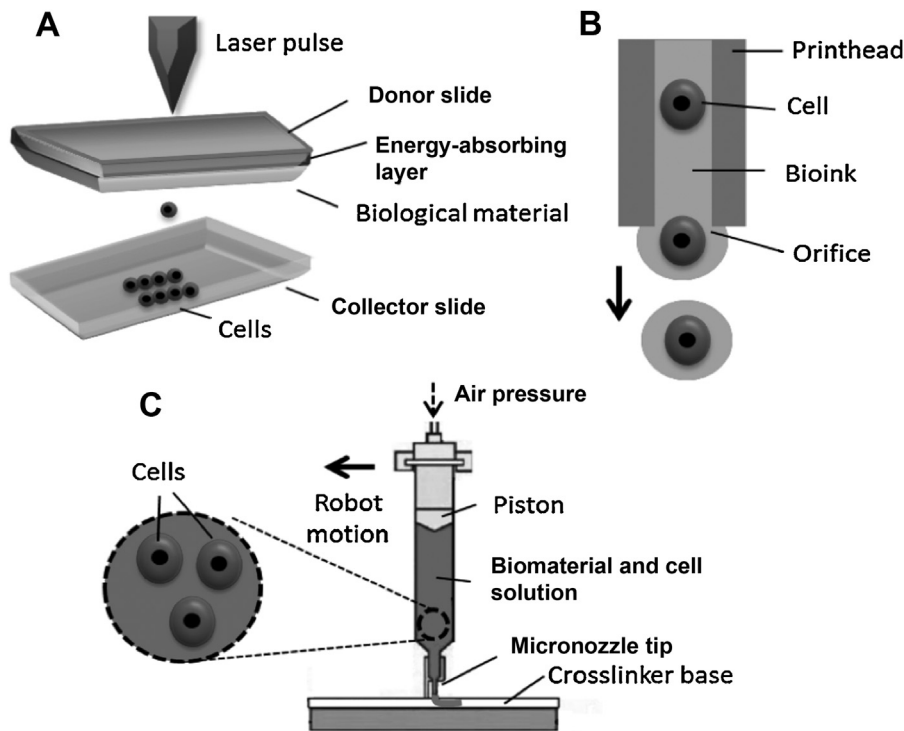
Bioinks are a key element for inkjet bioprinting where great attention is placed on minimizing cell settling and aggregation. An ideal bioink should exhibit acceptable cell viability while meeting the physical requirements necessary for printing (Derby, 2010). In a recent study by Ferris et al., a novel bioink was created and exhibited considerable cell viability, including highly sensitive neural cells after printing (Ferris et al., 2013). The bioink was composed of endotoxin-free low-acyl gellan gum, Milli-Q water, Dulbecco's modified Eagles medium, Poloxamer 188 surfactant, and/or fluoro-surfactant solution mixed with cells. Neural (PC-12) and skeletal muscle (C2C12) cells contained bioinks can maintain high cell viability over extended time periods without settling and aggregation.

Time-released delivery systems are another important feature that is highly desirable with regard to tissue engineered constructs. Growth factors incorporated within tissue substitutes can support and promote cell adhesion and proliferation during the initial period of implantation when endogenous growth factors are absent (Kokai, Bourbeau, Weber, Mcatee, & Marra, 2011; Lee et al., 2003). Lee et al. developed an artificial neural tissue containing vascular endothelial growth factor (VEGF) release by printing murine neural stem cells (C17.2), collagen, and fibrin gel together (Lee et al., 2010). C17.2 cells embedded within the collagen showed comparable viability ($92.89 \pm 2.32\%$) after printing with that of manually plated cells. When C17.2 cells were printed 1 mm from border of VEGF-releasing fibrin gel, the cells tended to migrate toward the fibrin gel. This work

**FIGURE 16.10**

(A) Schematic illustration of PEDOT/PSS bioink structure. (B–C) Electrical properties of scaffolds with different amount of PEDOT/PSS. The EG (ethylene glycol) supplement in the bioink improved the charge delivery capacity of scaffolds. (D) Electrical stimulation improved the neuronal differentiation of DRG cells in the scaffolds.

Adopted from Heo, D. N., Lee, S.-J., Timsina, R., Qiu, X., Castro, N. J., & Zhang, L. G. (2019). Development of 3D printable conductive hydrogel with crystallized PEDOT:PSS for neural tissue engineering. *Materials Science and Engineering: C*, 99, 582–590.

**FIGURE 16.11**

Typical 3D bioprinting techniques: (A) laser-based writing of cells, (B) inkjet-based systems, and (C) extrusion-based deposition.

Adopted from Ozbolat, I. T., & Yu, Y. (2013). Bioprinting toward organ fabrication: Challenges and future trends. IEEE Transactions on Bio-medical Engineering, 60, 691–699.

illustrates the capability of VEGF-containing fibrin gels for sustained release. Our laboratory developed a bioink by conjugating dopamine with gelatin methacrylate (GelMA). The dopamine is an essential neurotransmitter that can regulate neuronal development and enhance neurite outgrowth (Zhou et al., 2018). This bioink was used to 3D bioprint scaffolds containing neural stem cells. It was found that dopamine-conjugated scaffolds can promote neuronal differentiation of neural stem cells embedded in the scaffolds. These findings help to illustrate the role of sustained growth factor delivery within a 3D-bioprinted neural construct.

In addition to cellular level investigations, some studies have investigated the capacity of bioprinted grafts in recovering sensory and motor function in vivo. In a study published by Owens et al., a cellular nerve graft containing the analogous potential to an autologous graft was fabricated via bioprinting (Owens, Marga, Forgacs, & Heesch, 2013). Mouse bone marrow stem cells and Schwann cells were shaped into cylindrical units as bioink constituents and then printed layer-by-layer to form a neural graft. In vivo experiments demonstrated the cellular nerve graft performed satisfactorily in recovering both motor and sensory function. In a study by Zhu et al., a rapid

continuous 3D-printing platform was used to produce nerve guidance conduits with microchannels in an effort to direct new nerve branching and outgrowth (Zhu, Tringale, et al., 2018). Indeed, the implantation of this conduit into sciatic nerve transections of mouse models illustrated the effective directional guidance of newly grown sciatic nerves toward the distal end of the injury site. Meanwhile, animals receiving the conduit implantation presented promising recovery of motor function and sensation in their ipsilateral limbs.

Clinically, cells used for fabricating the cellular graft would be harvested from the patient thus preventing immunological rejection. With regard to defect size, bioprinting allows for the fabrication of clinically relevant grafts, and the present work confirmed the superiority of a bioprinted graft when compared to an autologous graft, the current therapeutic “gold standard” for nerve injuries.

16.3.2 4D BIOPRINTING FOR NEURAL TISSUE REGENERATION

As a future biomanufacturing technique, 4D printing was emerged by introducing an additional dimension of time to 3D printing. 4D-printed objects are capable of self-transforming in form or function when the external stimulations, such as heat, current, ultraviolet light, or other energy sources, are applied (Miao et al., 2017). 4D printing (known as 4D bioprinting when applied in biomedicine) was just started to be applied in the field of tissue engineering because it may replicate the natural biomechanical changes over time to mimic the dynamic feature occurring in natural tissue growth (Esworthy et al., 2019; Hann, Cui, Nowicki, & Zhang, 2020; Zhu et al., 2019).

Smart stimulus-responsive materials could be one of the essences of 4D printing that endow the capacity of printed objects to change architecture postprinting. A large number of such materials have been studied in neural regeneration. Our laboratory developed a novel multiresponsive 4D-bioprinted scaffold using a natural based, photocrosslinkable material named soybean oil epoxidized acrylate (Miao et al., 2018). In this platform, stress-induced shape transformation is employed to achieve the 4D reprogramming. Specifically, stereolithographic solidification induced internal stress to the material, which can be released by a subsequent solvent-induced relaxation to drive an autonomous, reversible, programmed configuration change postprinting (Fig. 16.12). Bioactive factors and various nanomaterials can be easily incorporated into the scaffolds. A proof-of-concept smart nerve guidance conduit generated by this 4D-printing system provided outstanding multifunctional features for nerve regeneration, such as physical and chemical cues, dynamic self-entubulation, and seamless integration (Miao et al., 2018).

One of the challenges of 4D bioprinting is to precisely control the shape-transformation. To solve this issue, our laboratory employed a facile 4D bioprinting platform that incorporated nanomaterials into the conventional stimuli-responsive polymer to achieve dynamic and precisely remote-controlled shape transformations (Cui et al., 2019). In this study, a novel near-infrared light (NIR) responsive 4D-bioprinted nanocomposite composed of a rigid epoxy monomer (bisphenol A diglycidyl ether), an aliphatic diamine crosslinker (poly(propylene glycol) bis(2-aminopropyl) ether), a cross-linking modulator [decylamine, which is used to tailor the transition temperature (T_g) of the polymers], and graphene (which can produce thermal energy by absorbing photons of NIR illumination) was developed. The printed objects with this platform can transfer their shapes via the “thermomechanical reprogramming” process when the glass temperature is higher than T_g of the material. In comparison to direct thermally triggered shape change, this light-switch

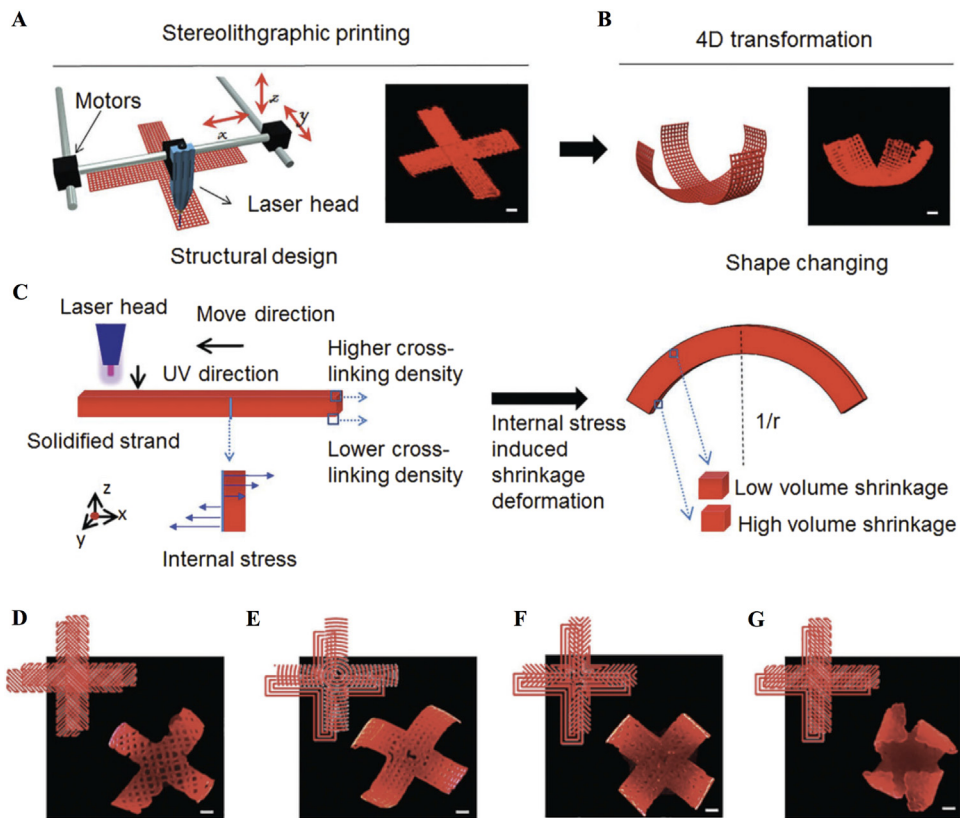


FIGURE 16.12

(A) Schematic illustration of the stereolithographic printing platform. (B) Illustration of the 4D transformation of printed scaffold poststimulation. (C) Illustration of the internal-stress-induced shape change. (D–G) Various 4D transformations depended on the different printing route designs.

Adopted from Miao, S., Cui, H., Nowicki, M., Xia, L., Zhou, X., Lee, S.-J., ... Zhang, L. G. (2018). Stereolithographic 4D bioprinting of multiresponsive architectures for neural engineering. *Advanced Biosystems*, 2, 1800101.

stimulation enables to achieve a remote, precise, and dynamic control over the time and position. Moreover, the NIR is benign energy to human which can penetrate to human tissues without biological harm. This is superior to many other stimuli. The neural stem cells-laden in the 4D-printed brain model by this platform presented excellent cell growth and differentiation. In another of our studies, the effects of dynamic transformation on 4D-bioprinted scaffolds on cell response have been investigated in detail (Miao et al., 2020). To this end, a 4D neural culture substrate was fabricated to replicate neural stem cell differentiation at different stages from cellular aggregation at an early stage to highly aligned micropatterns. It was found that the 4D culture substrate underwent a time-dependent self-morphing process that played a significant role in regulating neural stem cell behaviors in a spatiotemporal manner and enhanced neural differentiation along with improved

axonal alignment. These findings suggested dynamic smart substrates hold great potential to revolutionize current stem cell treatment, and may have a far-reaching impact on improving tissue regeneration, disease modeling, as well as other life science researches.

16.4 CONCLUSION AND FUTURE DIRECTIONS

This chapter reviewed recent advancements with regard to the use of nanotechnology and 3D/4D bioprinting as novel tools to fabricate bioactive constructs for improved neural regeneration. The intrinsic merit of nanotechnology to regenerative medicine is its high capacity to mimic the physical and chemical properties of natural ECM. A variety of self-assembling nanomaterials, carbon nanobiomaterials, and electrospun nanofibers currently under investigation cannot only provide a suitable nanoscale environment for neural cell adhesion and growth but also direct neural tissue repair and regeneration via topographical or chemical guidance. In addition, 3D bioprinting is an excellent approach for the fabrication of patient-specific complex neural grafts where various bioactive factors and cells can be readily incorporated and synergistically employed for improved neural regeneration. The evolution of 3D bioprinting to 4D bioprinting endows this technique more complexity to achieve dynamic morphological changes favorable to cell function under certain stimuli. Despite the vast improvements of nanotechnology and 3D/4D bioprinting in neural tissue engineering, the development of ideal neural grafts is still in its infancy. Currently, grafts lack the capacity to bridge defects >30 mm as well as address patients who suffer multiple traumatic injuries (Marquardt & Sakiyama-Elbert, 2013). In addition to spatial guidance of 3D neural constructs, controllable temporal release of growth factors is encouraged for future research. Moreover, current 3D/4D bioprinting techniques also face many challenges and their ultimate success for neural applications largely relies on the development of suitable biomaterials to be used as “inks” for the fabrication of robust neural tissue, especially the stimulus-responsive biomaterials for 4D bioprinting. Although various traditional biomaterials have continued to be improved, they have been limited by inadequate biomimetic properties which cannot satisfy the strict requirements for neural tissue regrowth. It is ideal to integrate 3D/4D bioprinting and biologically inspired nanobiomaterials that mimic native cellular and ECM for the next generation of neural tissue repair and regeneration.

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QUESTIONS

1. What are the key features that an ideal neural scaffold should possess?

2. What are the biological components that can self-assemble into nanostructures, and stimuli that can promote the occurrence of self-assembly?
3. Describe a few biomaterials used for electrospinning in neural tissue engineering application and the strategies used to improve the properties of electrospun neural scaffolds.
4. Describe a few popularly used carbon nanomaterials in neural tissue engineering, including their characteristics, and examples detailing how they have been used in neural tissue engineering.
5. What are the main 3D bioprinting techniques for neural tissue engineering?
6. What is the difference between 3D and 4D bioprinting as well as the merits of 4D bioprinting when compared to 3D bioprinting?
7. What is the biggest challenge of current 3D/4D bioprinting techniques for their ultimate success in neural tissue engineering applications?

References

- Abdul kafi, M., El-Said, W. A., Kim, T.-H., & Choi, J.-W. (2012). Cell adhesion, spreading, and proliferation on surface functionalized with RGD nanopillar arrays. *Biomaterials*, 33, 731–739.
- Aldinucci, A., Massacesi, L., Mello, T., Scaini, D., Bianco, A., Ballerini, L., ... Cavalieri, D. (2013). Carbon nanotube scaffolds instruct human dendritic cells: Modulating immune responses by contacts at the nano-scale. *Nano Letters*, 13, 6098–6105.
- Asheghali, D., Lee, S.-J., Furchner, A., Gruz, A., Larson, S., Tokarev, A., ... Minko, S. (2020). Enhanced neuronal differentiation of neural stem cells with mechanically enhanced touch-spun nanofibrous scaffolds. *Nanomedicine: Nanotechnology, Biology and Medicine*, 24, 102152.
- Balasubramanian, K., & Burghard, M. (2005). Chemically functionalized carbon nanotubes. *Small (Weinheim an der Bergstrasse, Germany)*, 1, 180–192.
- Banerjee, S., Hemraj-Benny, T., & Wong, S. S. (2005). Covalent surface chemistry of single-walled carbon nanotubes. *Advanced Materials*, 17, 17–29.
- Bei, H. P., Yang, Y., Zhang, Q., Tian, Y., Luo, X., Yang, M., & Zhao, X. (2019). Graphene-based nanocomposites for neural tissue engineering. *Molecules (Basel, Switzerland)*, 24, 658.
- Bekyarova, E., Ni, Y. C., Malarkey, E. B., Montana, V., McWilliams, J. L., Haddon, R. C., & Parpura, V. (2005). Applications of carbon nanotubes in biotechnology and biomedicine. *Journal of Biomedical Nanotechnology*, 1, 3–17.
- Brett Runge, M., Dadsetan, M., Baltrusaitis, J., Knight, A. M., Ruesink, T., Lazcano, E. A., ... Yaszemski, M. J. (2010). The development of electrically conductive polycaprolactone fumarate–polypyrrole composite materials for nerve regeneration. *Biomaterials*, 31, 5916–5926.
- Bruggeman, K. F., Rodriguez, A. L., Parish, C. L., Williams, R. J., & Nisbet, D. R. (2016). Temporally controlled release of multiple growth factors from a self-assembling peptide hydrogel. *Nanotechnology*, 27, 385102.
- Burger, C., Hsiao, B. S., & Chu, B. (2006). Nanofibrous materials and their applications. *Annual Review of Materials Research*, 36, 333–368.
- Bédier, A., Seichepine, F., Flahaut, E., Loubinoux, I., Vaysse, L., & Vieu, C. (2012). Elucidation of the role of carbon nanotube patterns on the development of cultured neuronal cells. *Langmuir: The ACS Journal of Surfaces and Colloids*, 28, 17363–17371.
- Cao, B., Li, Y., Yang, T., Bao, Q., Yang, M., & Mao, C. (2019). Bacteriophage-based biomaterials for tissue regeneration. *Advanced Drug Delivery Reviews*, 145, 73–95.

- Cao, H. Q., Liu, T., & Chew, S. Y. (2009). The application of nanofibrous scaffolds in neural tissue engineering. *Advanced Drug Delivery Reviews*, *61*, 1055–1064.
- Cheng, T.-Y., Chen, M.-H., Chang, W.-H., Huang, M.-Y., & Wang, T.-W. (2013). Neural stem cells encapsulated in a functionalized self-assembling peptide hydrogel for brain tissue engineering. *Biomaterials*, *34*, 2005.
- Childs, A., Hemraz, U. D., Castro, N. J., Fenniri, H., & Zhang, L. G. (2013). Novel biologically-inspired rosette nanotube PLLA scaffolds for improving human mesenchymal stem cell chondrogenic differentiation. *Biomedical Materials (Bristol, England)*, *8*, 065003.
- Christopherson, G. T., Song, H., & Mao, H.-Q. (2009). The influence of fiber diameter of electrospun substrates on neural stem cell differentiation and proliferation. *Biomaterials*, *30*, 556–564.
- Chronakis, I. S., Grapenson, S., & Jakob, A. (2006). Conductive polypyrrole nanofibers via electrospinning: Electrical and morphological properties. *Polymer*, *47*, 1597–1603.
- Constans, A. (2004). Neural tissue engineering. *Scientist (Philadelphia, Pa.)*, *18*, 40–42.
- Cui, H., Miao, S., Esworthy, T., Lee, S.-J., Zhou, X., Hann, S. Y., ... Zhang, L. G. (2019). A novel near-infrared light responsive 4D printed nanoarchitecture with dynamically and remotely controllable transformation. *Nano Research*, *12*, 1381–1388.
- Cui, H., Webber, M. J., & Stupp, S. I. (2010). Self-assembly of peptide amphiphiles: From molecules to nanostructures to biomaterials. *Biopolymers*, *94*, 1–18.
- Cunha, C., Panseri, S., & Antonini, S. (2011). Emerging nanotechnology approaches in tissue engineering for peripheral nerve regeneration. *Nanomedicine: Nanotechnology, Biology, and Medicine*, *7*, 50–59.
- Dahlin, R. L., Kasper, F. K., & Mikos, A. G. (2011). Polymeric nanofibers in tissue engineering. *Tissue Engineering. Part B, Reviews*, *17*, 349–364.
- Dai, H. (2002). Carbon nanotubes: Synthesis, integration, and properties. *Accounts of Chemical Research*, *35*, 1035–1044.
- Derby, B. (2010). Inkjet printing of functional and structural materials: Fluid property requirements, feature stability, and resolution. *Annual Review of Materials Research*, *40*, 395–414.
- Ellis-Behnke, R. G., Liang, Y. X., You, S. W., Tay, D. K., Zhang, S., So, K. F., & Schneider, G. E. (2006). Nano neuro knitting: Peptide nanofiber scaffold for brain repair and axon regeneration with functional return of vision. *Proceedings of the National Academy of Sciences of the United States of America*, *103*, 5054–5059.
- Esworthy, T. J., Miao, S., Lee, S.-J., Zhou, X., Cui, H., Zuo, Y. Y., & Zhang, L. G. (2019). Advanced 4D-bioprinting technologies for brain tissue modeling and study. *International Journal of Smart and Nano Materials*, *10*, 177–204.
- Fan, L., Feng, C., Zhao, W., Qian, L., Wang, Y., & Li, Y. (2012). Directional neurite outgrowth on superaligned carbon nanotube yarn patterned substrate. *Nano Letters*, *12*, 3668–3673.
- Ferris, C. J., Gilmore, K. J., Beirne, S., McCallum, D., Wallace, G. G., & Panhuis, M. I. H. (2013). Bio-ink for on-demand printing of living cells. *Biomaterials Science*, *1*, 224–230.
- Fine, E., Zhang, L., Fenniri, H., & Webster, T. J. (2009). Enhanced endothelial cell functions on rosette nanotube-coated titanium vascular stents. *International Journal of Nanomedicine*, *4*, 91–97.
- Fuhrer, M. S., Lau, C. N., & Macdonald, A. H. (2010). Graphene: Materially better carbon. *MRS Bulletin*, *35*, 289–295.
- Geim, A. K. (2009). Graphene: Status and prospects. *Science (New York, N.Y.)*, *324*, 1530–1534.
- Geim, A. K., & Novoselov, K. S. (2007). The rise of graphene. *Nature Materials*, *6*, 183–191.
- Gelain, F., Baldissera, F., Vescovi, A., Panseri, S., Antonini, S., Cunha, C., ... Montagna, M. (2011). Transplantation of nanostructured composite scaffolds results in the regeneration of chronically injured spinal cords. *ACS Nano*, *5*, 227–236.
- Gelain, F., Unsworth, L. D., & Zhang, S. G. (2010). Slow and sustained release of active cytokines from self-assembling peptide scaffolds. *Journal of Controlled Release*, *145*, 231–239.

- George, P. M., Sur, M., Lyckman, A. W., Lavan, D. A., Hegde, A., Leung, Y., ... Langer, R. (2005). Fabrication and biocompatibility of polypyrrole implants suitable for neural prosthetics. *Biomaterials*, 26, 3511–3519.
- Ghasemi-Mobarakeh, L., Prabhakaran, M. P., Morshed, M., Nasr-Esfahani, M. H., & Ramakrishna, S. (2009). Electrical stimulation of nerve cells using conductive nanofibrous scaffolds for nerve tissue engineering. *Tissue Engineering. Part A*, 15, 3605–3619.
- Gladwin, K. M., Whitby, R. L. D., Mikhalovsky, S. V., Tomlins, P., & Adu, J. (2013). In vitro biocompatibility of multiwalled carbon nanotubes with sensory neurons. *Advanced Healthcare Materials*, 2, 728–735.
- Gottipati, M. K., Samuelson, J. J., Kalinina, I., Bekyarova, E., Haddon, R. C., & Parpura, V. (2013). Chemically functionalized single-walled carbon nanotube films modulate the morpho-functional and proliferative characteristics of astrocytes. *Nano Letters*, 13, 4387.
- Guo, J., Su, H., Zeng, Y., Liang, Y.-X., Wong, W. M., Ellis-Behnke, R. G., ... Wu, W. (2007). Reknitting the injured spinal cord by self-assembling peptide nanofiber scaffold. *Nanomedicine: Nanotechnology, Biology, and Medicine*, 3, 311–321.
- Hann, S. Y., Cui, H., Nowicki, M., & Zhang, L. G. (2020). 4D printing soft robotics for biomedical applications. *Additive Manufacturing*, 36, 101567.
- Hauser, C. A. E., & Zhang, S. (2010). Designer self-assembling peptide nanofiber biological materials. *Chemical Society Reviews*, 39, 2780–2790.
- Heo, D. N., Lee, S.-J., Timsina, R., Qiu, X., Castro, N. J., & Zhang, L. G. (2019). Development of 3D printable conductive hydrogel with crystallized PEDOT:PSS for neural tissue engineering. *Materials Science and Engineering: C*, 99, 582–590.
- Hirsch, A. (2002). Functionalization of single-walled carbon nanotubes. *Angewandte Chemie (International ed.) in English*, 41, 1853–1859.
- Hosseinkhani, H., Hong, P.-D., & Yu, D.-S. (2013). Self-assembled proteins and peptides for regenerative medicine. *Chemical Reviews*, 113, 4837–4861.
- Hu, H., Ni, Y., Montana, V., Haddon, R. C., & Parpura, V. (2004). Chemically functionalized carbon nanotubes as substrates for neuronal growth. *Nano Letters*, 4, 507–511.
- Hu, J., Tian, L., Prabhakaran, M. P., Ding, X., & Ramakrishna, S. (2016). Fabrication of nerve growth factor encapsulated aligned poly(ϵ -caprolactone) nanofibers and their assessment as a potential neural tissue engineering scaffold. *Polymers*, 8, 54.
- Huang, J., Lu, L., Zhang, J., Hu, X., Zhang, Y., Liang, W., ... Luo, Z. (2012). Electrical stimulation to conductive scaffold promotes axonal regeneration and remyelination in a rat model of large nerve defect. *PLoS One*, 7, e39526.
- Iwasaki, M., Wilcox, J. T., Nishimura, Y., Zweckberger, K., Suzuki, H., Wang, J., ... Fehlings, M. G. (2014). Synergistic effects of self-assembling peptide and neural stem/progenitor cells to promote tissue repair and forelimb functional recovery in cervical spinal cord injury. *Biomaterials*, 35, 2617.
- Jan, E., & Kotov, N. A. (2007). Successful differentiation of mouse neural stem cells on layer-by-layer assembled single-walled carbon nanotube composite. *Nano Letters*, 7, 1123–1128.
- Jin, L., Feng, Z. Q., Zhu, M. L., Wang, T., Leach, M. K., & Jiang, Q. (2012). A novel fluffy conductive polypyrrole nano-layer coated PLLA fibrous scaffold for nerve tissue engineering. *Journal of Biomedical Nanotechnology*, 8, 779–785.
- Katsnelson, M. I. (2007). Graphene: Carbon in two dimensions. *Materials Today*, 10, 20–27.
- Keefer, E. W., Botterman, B. R., Romero, M. I., Rossi, A. F., & Gross, G. W. (2008). Carbon nanotube coating improves neuronal recordings. *Nature Nanotechnology*, 3, 434–439.
- Keun Kwon, I., Kidoaki, S., & Matsuda, T. (2005). Electrospun nano- to microfiber fabrics made of biodegradable copolyesters: Structural characteristics, mechanical properties and cell adhesion potential. *Biomaterials*, 26, 3929–3939.

- Kim, Y. G., Lee, Y. I., Kim, J. W., Pyeon, H. J., Hyun, J. K., Hwang, J.-Y., ... Kim, H.-W. (2014). Differential stimulation of neurotrophin release by the biocompatible nano-material (carbon nanotube) in primary cultured neurons. *Journal of Biomaterials Applications*, 28, 790.
- Ko, S. H., Su, M., Zhang, C., Ribbe, A. E., Jiang, W., & Mao, C. (2010). Synergistic self-assembly of RNA and DNA molecules. *Nature Chemistry*, 2, 1050–1055.
- Kokai, L. E., Bourbeau, D., Weber, D., Mcatee, J., & Marra, K. G. (2011). Sustained growth factor delivery promotes axonal regeneration in long gap peripheral nerve repair. *Tissue Engineering. Part A*, 17, 1263–1275.
- Kumar, R., Aadil, K. R., Ranjan, S., & Kumar, V. B. (2020). Advances in nanotechnology and nanomaterials based strategies for neural tissue engineering. *Journal of Drug Delivery Science and Technology*, 57, 101617.
- Kumbar, S. G., James, R., Nukavarapu, S. P., & Laurencin, C. T. (2008). Electrospun nanofiber scaffolds: Engineering soft tissues. *Biomedical Materials*, 3, 034002.
- Lee, A. C., Yu, V. M., Lowe, J. B., Brenner, M. J., Hunter, D. A., Mackinnon, S. E., & Sakiyama-Elbert, S. E. (2003). Controlled release of nerve growth factor enhances sciatic nerve regeneration. *Experimental Neurology*, 184, 295–303.
- Lee, J. Y., Bashur, C. A., Goldstein, A. S., & Schmidt, C. E. (2009). Polypyrrole-coated electrospun PLGA nanofibers for neural tissue applications. *Biomaterials*, 30, 4325–4335.
- Lee, S.-J., Asheghali, D., Blevins, B., Timsina, R., Esworthy, T., Zhou, X., ... Zhang, L. G. (2020). Touch-spun nanofibers for nerve regeneration. *ACS Applied Materials & Interfaces*, 12, 2067–2075.
- Lee, S.-J., Zhu, W., Nowicki, M., Lee, G., Heo, D. N., Kim, J., ... Zhang, L. G. (2018). 3D printing nano conductive multi-walled carbon nanotube scaffolds for nerve regeneration. *Journal of Neural Engineering*, 15, 016018.
- Lee, W., Lee, Y.-B., Polio, S., Dai, G., Menon, L., Carroll, R. S., & Yoo, S.-S. (2010). Bio-printing of collagen and VEGF-releasing fibrin gel scaffolds for neural stem cell culture. *Experimental Neurology*, 223, 645–652.
- Li, J., Xing, R., Bai, S., & Yan, X. (2019). Recent advances of self-assembling peptide-based hydrogels for biomedical applications. *Soft Matter*, 15, 1704–1715.
- Li, N., Cheng, G., Zhang, Q., Gao, S., Song, Q., Huang, R., ... Tang, M. (2013). Three-dimensional graphene foam as a biocompatible and conductive scaffold for neural stem cells. *Scientific Reports*, 3, 1604.
- Liang, Y.-X., Cheung, S. W. H., Chan, K. C. W., Wu, E. X., Tay, D. K. C., & Ellis-Behnke, R. G. (2011). CNS regeneration after chronic injury using a self-assembled nanomaterial and MEMRI for real-time in vivo monitoring. *Nanomedicine: Nanotechnology, Biology, and Medicine*, 7, 351–359.
- Liu, X., Miller, A. L., Park, S., Waletzki, B. E., Zhou, Z., Terzic, A., & Lu, L. (2017). Functionalized carbon nanotube and graphene oxide embedded electrically conductive hydrogel synergistically stimulates nerve cell differentiation. *ACS Applied Materials & Interfaces*, 9, 14677–14690.
- Liu, Y., Ye, H., Satkunendrarajah, K., Yao, G. S., Bayon, Y., & Fehlings, M. G. (2013). A self-assembling peptide reduces glial scarring, attenuates post-traumatic inflammation and promotes neurological recovery following spinal cord injury. *Acta Biomaterialia*, 9, 8075–8088.
- Lock, L. L., Lacombe, M., Schwarz, K., Cheetham, A. G., Lin, Y.-A., Zhang, P., & Cui, H. (2013). Self-assembly of natural and synthetic drug amphiphiles into discrete supramolecular nanostructures. *Faraday Discussions*, 166, 285–301.
- Lu, J., Sun, X., Yin, H., Shen, X., Yang, S., Wang, Y., ... Wang, X. (2018). A neurotrophic peptide-functionalized self-assembling peptide nanofiber hydrogel enhances rat sciatic nerve regeneration. *Nano Research*, 11, 4599–4613.
- Ma, Z., Kotaki, M., Inai, R., & Ramakrishna, S. (2005). Potential of nanofiber matrix as tissue-engineering scaffolds. *Tissue Engineering*, 11, 101–109.
- Malarkey, E. B., Fisher, K. A., Bekyarova, E., Liu, W., Haddon, R. C., & Parpura, V. (2009). Conductive single-walled carbon nanotube substrates modulate neuronal growth. *Nano Letters*, 9, 264–268.
- Marchesan, S., Ballerini, L., & Prato, M. (2017). Nanomaterials for stimulating nerve growth. *Science (New York, N.Y.)*, 356, 1010.

- Marquardt, L. M., & Sakiyama-Elbert, S. E. (2013). Engineering peripheral nerve repair. *Current Opinion in Biotechnology*, 24, 887–892.
- Matson, J. B., & Stupp, S. I. (2011). Self-assembling peptide scaffolds for regenerative medicine. *Chemical Communications (Cambridge, England)*, 48, 26–33.
- Matsumoto, K., Sato, C., Naka, Y., Whitby, R., & Shimizu, N. (2010). Stimulation of neuronal neurite outgrowth using functionalized carbon nanotubes. *Nanotechnology*, 21, 115101.
- Maude, S., Ingham, E., & Aggeli, A. (2013). Biomimetic self-assembling peptides as scaffolds for soft tissue engineering. *Nanomedicine: Nanotechnology, Biology, and Medicine*, 8, 823–847.
- Melchels, F. P. W., Domingos, M. A. N., Klein, T. J., Malda, J., Bartolo, P. J., & Huttmacher, D. W. (2012). Additive manufacturing of tissues and organs. *Progress in Polymer Science*, 37, 1079–1104.
- Melissinaki, V., Gill, A.A., Ortega, I., Vamvakaki, M., Ranella, A., Fotakis, C., ... Claeysens, F. Direct laser writing of polylactide 3D scaffolds for neural tissue engineering applications. In *Conference on lasers and electro-optics Europe and 12th European quantum electronics conference (CLEO EUROPE/EQEC)*, 2011, p. 1, doi: 10.1109/CLEOE.2011.5943316. IEEE.
- Merzlyak, A., Indrakanti, S., & Lee, S.-W. (2009). Genetically engineered nanofiber-like viruses for tissue regenerating materials. *Nano Letters*, 9, 846–852.
- Miao, S., Castro, N., Nowicki, M., Xia, L., Cui, H., Zhou, X., ... Zhang, L. G. (2017). 4D printing of polymeric materials for tissue and organ regeneration. *Materials Today*, 20, 577–591.
- Miao, S., Cui, H., Esworthy, T., Mahadik, B., Lee, S.-J., Zhou, X., ... Zhang, L. G. (2020). 4D self-morphing culture substrate for modulating cell differentiation. *Advanced Science*, 7, 1902403.
- Miao, S., Cui, H., Nowicki, M., Xia, L., Zhou, X., Lee, S.-J., ... Zhang, L. G. (2018). Stereolithographic 4D bioprinting of multiresponsive architectures for neural engineering. *Advanced Biosystems*, 2, 1800101.
- Michael, C. D., Abbas, R., Saksham, G., Ronnie, E. B., Ya-Ching, H., Maria, P., ... Kee, B. P. (2019). Estimating the global incidence of traumatic brain injury. *Journal of Neurosurgery JNS*, 130, 1080–1097.
- Ni, Y., Hu, I., Malarkey, E. B., Zhao, B., Montana, V., Haddon, R. C., & Parpura, V. (2005). Chemically functionalized water soluble single-walled carbon nanotubes modulate neurite outgrowth. *Journal of Nanoscience and Nanotechnology*, 5, 1707–1712.
- Niu, Y., Chen, X., Yao, D., Peng, G., Liu, H., & Fan, Y. (2018). Enhancing neural differentiation of induced pluripotent stem cells by conductive graphene/silk fibroin films. *Journal of Biomedical Materials Research. Part A*, 106, 2973–2983.
- Owens, C. M., Marga, F., Forgacs, G., & Heesch, C. M. (2013). Biofabrication and testing of a fully cellular nerve graft. *Biofabrication*, 5, 045007.
- Ozbolat, I. T., & Yu, Y. (2013). Bioprinting toward organ fabrication: Challenges and future trends. *IEEE Transactions on Bio-medical Engineering*, 60, 691–699.
- Panseri, S., Cunha, C., Lowery, J., Del carro, U., Taraballi, F., Amadio, S., ... Gelain, F. (2008). Electrospun micro- and nanofiber tubes for functional nervous regeneration in sciatic nerve transections. *BMC Biotechnology*, 8, 39.
- Park, H.-B., Nam, H.-G., Oh, H.-G., Kim, J.-H., Kim, C.-M., Song, K.-S., & Jhee, K.-H. (2013). Effect of graphene on growth of neuroblastoma cells. *Journal of Microbiology and Biotechnology*, 23, 274–277.
- Park, S. Y., Choi, D. S., Jin, H. J., Park, J., Byun, K.-E., Lee, K.-B., & Hong, S. (2011). Polarization-controlled differentiation of human neural stem cells using synergistic cues from the patterns of carbon nanotube monolayer coating. *ACS Nano*, 5, 4704–4711.
- Park, S. Y., Kang, B.-S., & Hong, S. (2013). Improved neural differentiation of human mesenchymal stem cells interfaced with carbon nanotube scaffolds. *Nanomedicine: Nanotechnology, Biology, and Medicine*, 8, 715–723.
- Peng, X. H., & Wong, S. S. (2009). Functional covalent chemistry of carbon nanotube surfaces. *Advanced Materials*, 21, 625–642.

- Posten, W., Wrone, D. A., Dover, J. S., Arndt, K. A., Silapunt, S., & Alam, M. (2005). Low-level laser therapy for wound healing: Mechanism and efficacy. *Dermatologic Surgery*, 31, 334–340.
- Puzan, M. L., Legesse, B., Koppes, R. A., Fenniri, H., & Koppes, A. N. (2018). Bioactive organic rosette nanotubes support sensory neurite outgrowth. *ACS Biomaterials Science & Engineering*, 4, 1630–1640.
- Qiu, F., Chen, Y., Tang, C., & Zhao, X. (2018). Amphiphilic peptides as novel nanomaterials: Design, self-assembly and application. *International Journal of Nanomedicine*, 13, 5003–5022.
- Rao, C. N. R., Sood, A. K., Subrahmanyam, K. S., & Govindaraj, A. (2009). Graphene: The new two-dimensional nanomaterial. *Angewandte Chemie (International ed.) in English*, 48, 7752–7777.
- Raspa, A., Carminati, L., Pugliese, R., Fontana, F., & Gelain, F. (2020). Self-assembling peptide hydrogels for the stabilization and sustained release of active Chondroitinase ABC in vitro and in spinal cord injuries. *Journal of Controlled Release*. Available from <https://doi.org/10.1016/j.jconrel.2020.11.027>.
- Reddy, S., He, L., Ramakrishana, S., & Luo, H. (2019). Graphene nanomaterials for regulating stem cell fate in neurogenesis and their biocompatibility. *Current Opinion in Biomedical Engineering*, 10, 69–78.
- Reddy, S., Xu, X., Guo, T., Zhu, R., He, L., & Ramakrishana, S. (2018). Allotropic carbon (graphene oxide and reduced graphene oxide) based biomaterials for neural regeneration. *Current Opinion in Biomedical Engineering*, 6, 120–129.
- Sabra, S., Ragab, D. M., Agwa, M. M., & Rohani, S. (2020). Recent advances in electrospun nanofibers for some biomedical applications. *European Journal of Pharmaceutical Sciences*, 144, 105224.
- Sadeghi, A., Mozarzadeh, F., & Aghazadeh Mohandesi, J. (2019). Investigating the effect of chitosan on hydrophilicity and bioactivity of conductive electrospun composite scaffold for neural tissue engineering. *International Journal of Biological Macromolecules*, 121, 625–632.
- Saito, N., Kato, H., Taruta, S., Endo, M., Usui, Y., Aoki, K., ... Ishigaki, N. (2009). Carbon nanotubes: Biomaterial applications. *Chemical Society Reviews*, 38, 1897–1903.
- Sanjana, N. E., & Fuller, S. B. (2004). A fast flexible ink-jet printing method for patterning dissociated neurons in culture. *Journal of Neuroscience Methods*, 136, 151–163.
- Saracino, G. A. A., Cigognini, D., Silva, D., Caprini, A., & Gelain, F. (2013). Nanomaterials design and tests for neural tissue engineering. *Chemical Society Reviews*, 42, 225–262.
- Schmidt, C. E., & Leach, J. B. (2003). Neural tissue engineering: Strategies for repair and regeneration. *Annual Review of Biomedical Engineering*, 5, 293–347.
- Shepherd, J. N. H., Parker, S. T., Shepherd, R. F., Gillette, M. U., Lewis, J. A., & Nuzzo, R. G. (2011). 3D microperiodic hydrogel scaffolds for robust neuronal cultures. *Advanced Functional Materials*, 21, 47–54.
- Shoichet, M., & Schmidt, H. C. (2001). Neural tissue engineering. *Biomaterials*, 22, 1015–1193.
- Shoichet, M. S., Tate, C. C., Douglas Baumann, M., & Laplaca, M. C. (2008). Strategies for regeneration and repair in the injured central nervous system. In W. M. Reichert (Ed.), *Indwelling neural implants: Strategies for contending with the in vivo environment*. CRC Press.
- Sill, T. J., & von Recum, H. A. (2008). Electrospinning: Applications in drug delivery and tissue engineering. *Biomaterials*, 29, 1989–2006.
- Smith, K. H., Tejeda-Montes, E., Poch, M., & Mata, A. (2011). Integrating top-down and self-assembly in the fabrication of peptide and protein-based biomedical materials. *Chemical Society Reviews*, 40, 4563.
- Stephanopoulos, N., Ortony, J. H., & Stupp, S. I. (2013). Self-assembly for the synthesis of functional biomaterials. *Acta Materialia*, 61, 912–930.
- Subramanian, A., Krishnan, U. M., & Sethuraman, S. (2009). Development of biomaterial scaffold for nerve tissue engineering: Biomaterial mediated neural regeneration. *Journal of Biomedical Science*, 16, 108.
- Sun, L., Zhang, L., Hemraz, U. D., Fenniri, H., & Webster, T. J. (2012). Bioactive rosette nanotube-hydroxyapatite nanocomposites improve osteoblast functions. *Tissue Engineering. Part A*, 18, 1741–1750.
- Sun, Y., Li, W., Wu, X., Zhang, N., Zhang, Y., Ouyang, S., ... Wu, W. (2016). Functional self-assembling peptide nanofiber hydrogels designed for nerve degeneration. *ACS Applied Materials & Interfaces*, 8, 2348–2359.

- Tang, M., Song, Q., Li, N., Jiang, Z., Huang, R., & Cheng, G. (2013). Enhancement of electrical signaling in neural networks on graphene films. *Biomaterials*, 34, 6402–6411.
- Theron, S. A., Zussman, E., & Yarin, A. L. (2004). Experimental investigation of the governing parameters in the electrospinning of polymer solutions. *Polymer*, 45, 2017–2030.
- Tokarev, A., Asheghali, D., Griffiths, I. M., Trotsenko, O., Gruzd, A., Lin, X., . . . Minko, S. (2015). Touch- and brush-spinning of nanofibers. *Advanced Materials*, 27, 6526–6532.
- Tran, P. A., Zhang, L., & Webster, T. J. (2009). Carbon nanofibers and carbon nanotubes in regenerative medicine. *Advanced Drug Delivery Reviews*, 61, 1097–1114.
- Tysseling, V. M., Sahni, V., Niece, K. L., Birch, D., Czeisler, C., Fehlings, M. G., . . . Kessler, J. A. (2008). Self-assembling nanofibers inhibit glial scar formation and promote axon elongation after spinal cord injury. *Journal of Neuroscience*, 28, 3814–3823.
- Tysseling, V. M., Sahni, V., Pashuck, E. T., Birch, D., Hebert, A., Czeisler, C., . . . Kessler, J. A. (2010). Self-assembling peptide amphiphile promotes plasticity of serotonergic fibers following spinal cord injury. *Journal of Neuroscience Research*, 88, 3161–3170.
- Vasita, R., & Katti, D. S. (2006). Nanofibers and their applications in tissue engineering. *International Journal of Nanomedicine*, 1, 15–30.
- Vasita, R., Shanmugam, K., & Katti, D. S. (2008). Improved biomaterials for tissue engineering applications: Surface modification of polymers. *Current Topics in Medicinal Chemistry*, 8, 341–353.
- Wang, J., Cheng, Y., Chen, L., Zhu, T., Ye, K., Jia, C., . . . Mo, X. (2019). In vitro and in vivo studies of electroactive reduced graphene oxide-modified nanofiber scaffolds for peripheral nerve regeneration. *Acta Biomaterialia*, 84, 98–113.
- Wang, Y., Wang, J., Li, Z., Li, J., & Lin, Y. (2011). Graphene and graphene oxide: Biofunctionalization and applications in biotechnology. *Trends in Biotechnology*, 29, 205–212.
- Wei, G.-J., Yao, M., Wang, Y.-S., Zhou, C.-W., Wan, D.-Y., Lei, P.-Z., . . . Dong, D.-M. (2013). Promotion of peripheral nerve regeneration of a peptide compound hydrogel scaffold. *International Journal of Nanomedicine*, 8, 3217–3225.
- Weng, B., Liu, X., Shepherd, R., & Wallace, G. G. (2012). Inkjet printed polypyrrole/collagen scaffold: A combination of spatial control and electrical stimulation of PC12 cells. *Synthetic Metals*, 162, 1375–1380.
- Xie, J., Macewan, M. R., Li, X., Sakiyama-Elbert, S. E., & Xia, Y. (2009). Neurite outgrowth on nanofiber scaffolds with different orders, structures, and surface properties. *ACS Nano*, 3, 1151–1159.
- Xie, J., Macewan, M. R., Schwartz, A. G., & Xia, Y. (2010). Electrospun nanofibers for neural tissue engineering. *Nanoscale*, 2, 35–44.
- Xu, C., & Kopeček, J. (2007). Self-assembling hydrogels. *Polymer Bulletin*, 58, 53–63.
- Xu, H., Holzwarth, J. M., Yan, Y., Xu, P., Zheng, H., Yin, Y., . . . Ma, P. X. (2014). Conductive PPY/PDLLA conduit for peripheral nerve regeneration. *Biomaterials*, 35, 225.
- Yang, S., Wang, C., Zhu, J., Lu, C., Li, H., Chen, F., . . . Wang, X. (2020). Self-assembling peptide hydrogels functionalized with LN- and BDNF-mimicking epitopes synergistically enhance peripheral nerve regeneration. *Theranostics*, 10, 8227–8249.
- Yang, W., Thordarson, P., Gooding, J. J., Ringer, S. P., & Braet, F. (2007). Carbon nanotubes for biological and biomedical applications. *Nanotechnology*, 18, 412001.
- Yang, Y., Asiri, A. M., Tang, Z., Du, D., & Lin, Y. (2013). Graphene based materials for biomedical applications. *Materials Today*, 16, 365.
- Yang, Y., Zhao, Z., Zhang, F., Nangreave, J., Liu, Y., & Yan, H. (2013). Self-assembly of DNA rings from scaffold-free DNA tiles. *Nano Letters*, 13, 1862–1866.
- Yang, Y. L., Khoe, U., Wang, X. M., Horii, A., Yokoi, H., & Zhang, S. G. (2009). Designer self-assembling peptide nanomaterials. *Nano Today*, 4, 193–210.

- Zamani, F., Amani-Tehran, M., Zaminy, A., & Shokrgozar, M.-A. (2017). Conductive 3D structure nanofibrous scaffolds for spinal cord regeneration. *Fibers and Polymers*, *18*, 1874–1881.
- Zhan, X., Zhang, W., Guo, J., Wu, W., Gao, M., Jiang, Y., . . . Dai, X. (2013). Nanofiber scaffolds facilitate functional regeneration of peripheral nerve injury. *Nanomedicine: Nanotechnology, Biology, and Medicine*, *9*, 305–315.
- Zhang, L., Hemraz, U. D., Fenniri, H., & Webster, T. J. (2010). Tuning cell adhesion on titanium with osteogenic rosette nanotubes. *Journal of Biomedical Materials Research. Part A*, *95*, 550–563.
- Zhang, L., Rakotondradany, F., Myles, A. J., Fenniri, H., & Webster, T. J. (2009). Arginine-glycine-aspartic acid modified rosette nanotube-hydrogel composites for bone tissue engineering. *Biomaterials*, *30*, 1309–1320.
- Zhang, L., Ramsaywack, S., Fenniri, H., & Webster, T. J. (2008). Enhanced osteoblast adhesion on self-assembled nanostructured hydrogel scaffolds. *Tissue Engineering. Part A*, *14*, 1353–1364.
- Zhang, L., Rodriguez, J., Raez, J., Myles, A. J., Fenniri, H., & Webster, T. J. (2009). Biologically inspired rosette nanotubes and nanocrystalline hydroxyapatite hydrogel nanocomposites as improved bone substitutes. *Nanotechnology*, *20*, 175101.
- Zhang, L. J., & Webster, T. J. (2009). Nanotechnology and nanomaterials: Promises for improved tissue regeneration. *Nano Today*, *4*, 66–80.
- Zhang, X., Prasad, S., Niyogi, S., Morgan, A., Ozkan, C. S., & Ozkan, M. (2005). Guided neurite growth on patterned carbon nanotubes. *Sensors & Actuators: B. Chemical*, *106*, 843–850.
- Zhang, X., Xin, N., Tong, L., & Tong, X.-J. (2013). Electrical stimulation enhances peripheral nerve regeneration after crush injury in rats. *Molecular Medicine Reports*, *7*, 1523–1527.
- Zhou, X., Cui, H., Nowicki, M., Miao, S., Lee, S.-J., Masood, F., . . . Zhang, L. G. (2018). Three-dimensional-bioprinted dopamine-based matrix for promoting neural regeneration. *ACS Applied Materials & Interfaces*, *10*, 8993–9001.
- Zhou, X., Tenaglio, S., Esworthy, T., Hann, S. Y., Cui, H., Webster, T. J., . . . Zhang, L. G. (2020). Three-dimensional printing biologically inspired DNA-based gradient scaffolds for cartilage tissue regeneration. *ACS Applied Materials & Interfaces*, *12*, 33219–33228.
- Zhu, W., George, J. K., Sorger, V. J., & Grace Zhang, L. (2017). 3D printing scaffold coupled with low level light therapy for neural tissue regeneration. *Biofabrication*, *9*, 025002.
- Zhu, W., Tringale, K. R., Woller, S. A., You, S., Johnson, S., Shen, H., . . . Chen, S. (2018). Rapid continuous 3D printing of customizable peripheral nerve guidance conduits. *Materials Today*, *21*, 951–959.
- Zhu, W., Webster, T. J., & Zhang, L. G. (2019). 4D printing smart biosystems for nanomedicine. *Nanomedicine: Nanotechnology, Biology, and Medicine*, *14*, 1643–1645.
- Zhu, W., Ye, T., Lee, S.-J., Cui, H., Miao, S., Zhou, X., . . . Hang, L. G. (2018). Enhanced neural stem cell functions in conductive annealed carbon nanofibrous scaffolds with electrical stimulation. *Nanomedicine: Nanotechnology, Biology and Medicine*, *14*, 2485–2494.

3D Bioprinting for Liver Regeneration

17

Sushila Maharjan, Diana Bonilla and Yu Shrike Zhang

Division of Engineering of Medicine, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Cambridge, MA, United States

17.1 INTRODUCTION

Since the early 2000s, the deaths related to liver diseases have risen significantly, causing approximately 2 million deaths per year globally (Sapanlou et al., 2020). The combination of the numbers of patients affected by cirrhosis and liver cancer alone account for 3.5% of the deaths, and they are currently the 11th and 16th most common causes of deaths, respectively (Asrani, Devarbhavi, Eaton, & Kamath, 2019). The cases of deaths due to liver diseases also take into consideration those of acute hepatitis resulting from viral infection and further disorders related to alcohol abuse or diet habits. Also, there is a considerable distinction between the Western highly industrialized countries that show a higher rate of affections attributed to nonalcoholic fatty liver disease (NAFLD) and Asian countries with a greater percentage of deaths due to hepatitis B (El-Serag, 2012). If detected in a timely manner, most of the liver disorders can be treated with antiviral drugs, lifestyle changes of healthy diet, exercises, and suspension of alcohol consumption, except from liver cancer that has to be assessed on a case-to-case basis (Hajdarevic, Vehabovic, Catic, & Masic, 2020). However, in the advanced stages of diseases that lead to partial or complete liver failure, the best chance to increment life expectancy is by liver transplantation. While the need for tissue allografts is increasing constantly, there is still a shortage of donors. In Asia, the number of living donations has expanded, while it has remained low in the Western hemisphere and the demand for liver transplants is not met, even if the surgical methods have become crucially more specific (Jadlowiec & Taner, 2016).

When a liver transplant is performed, there is still the probability of organ failure and renal damage after a prolonged time period due to the constant use of immunosuppressive drugs, besides that the tissue can suffer damage depending on the preserving methods before transplantation (Jadlowiec & Taner, 2016). Therefore the challenge to reduce immune rejection and maintain the healthy tissues in *ex vivo* conditions is still relevant. Due to the increasing necessity of healthy liver grafts that can trigger regeneration in patients who suffer from liver diseases, the tissue engineering area has researched various techniques with the aim of developing a viable construct that can potentially serve as a regenerative aid. Consequently, the focus of this chapter is to address current three-dimensional (3D) bioprinting technologies that are being used to develop liver tissue constructs, their structural and functional features, as well as their applications *in vitro* and *in vivo*.

17.2 STRUCTURAL AND FUNCTIONAL COMPLEXITY OF THE LIVER

The liver is the largest triangular-shaped solid organ in the body and is one of the most essential organs in the human system serving several critical functions. It has a significant contribution to the metabolism of food since it plays a crucial role in glycolysis and gluconeogenesis and insulin-related pathways. In the same way, it plays a central role in the absorption of lipids and cholesterol usage in hepatocytes through the liver's biliary system (Trefts, Gannon, & Wasserman, 2017). Furthermore, the liver also accomplishes the biotransformation of xenobiotics from lipophilic to hydrophilic compounds, so that they can be excreted from the body. This mechanism too has an implication in the absorption of drugs due to the first-pass metabolic action of the liver over pharmaceutical compounds. In fact, the liver is the primary site of drug metabolism (Almazroo, Miah, & Venkataramanan, 2017). Other important functions of the liver include bilirubin metabolism (Stec et al., 2016), regulation of blood clotting (Shah & Caldwell, 2016), blood filtration (Balmer et al., 2014), and storage of vitamins such as vitamins A, D, E, K, and B12, as well as minerals such as iron and copper (Kozeniecki, Ludke, Kerner, & Patterson, 2020).

The main structure of the liver is given by the blood supply it receives from the portal vein (approximately 75%) and the hepatic artery (approximately 25%) as approximately 1.5 L of blood enter the liver every minute (Kang & Elia, 2016). The liver is the only organ that receives dual blood supply from the portal vein and the hepatic artery (Gabriel, Maroney, & Ringe, 2007). The functional unit of the liver is the lobule, which is the hexagonal 3D structure with a central vein and a portal triad (portal vein, a hepatic artery, and a bile duct) in each corner (Saxena, Theise, & Crawford, 1999). An average dimension of a lobule would be 1 mm from an end vertex to a contrary side and around 2 mm of thickness (Zhang et al., 2017). In each of the liver lobules, a complex architecture is sustained by stratified heterogeneous cells that allow the correct performance of the organ. The most abundant types of cells are the hepatocytes forming the parenchymal tissue; they comprise around 80% of the total cell population (Ben-Moshe & Itzkovitz, 2019; Fausto & Campbell, 2003). The exchange of substances between the hepatocytes and the venous or arterial blood is facilitated by the multiple vessels that are part of the lobule's main structure (Schulze, Schott, Casey, Tuma, & McNiven, 2019). It is also aided by the apical and basolateral or sinusoidal membrane domains it possesses, which show a different composition of receptors, proteins, and channels between each other. This characteristic is known as hepatocyte polarity because the basal membrane interacts with the sinusoidal epithelial cells, and meanwhile the apical poles face the canaliculi for bile transport (Gissen & Arias, 2015). Although all hepatocytes are morphologically similar, the hepatocytes that surround the portal triad have different functions given their proximity to the vascular tissue and are specified into three metabolic functional areas known as "liver zonation" (Kietzmann, 2017). The periportal Zone 1 comprising six to eight hepatocyte layers receive blood enriched in oxygen and nutrients and thus periportal hepatocytes are responsible for oxidative liver functions such as gluconeogenesis, β -oxidation of fatty acids, ammonia detoxification, and cholesterol synthesis. Intermediate perivenous Zone 2 comprises 6–10 hepatocyte layers and perivenous hepatocytes play important role in xenobiotic metabolism. The pericentral (centrilobular) Zone 3 around the central vein comprises two to three hepatocyte layers and these pericentral hepatocytes perform important functions such as glycolysis, lipogenesis, bile acid synthesis, glutamine synthesis, and cytochrome P450-based drug detoxification (Casotti & D'Antiga, 2019; Jungermann & Katz, 1989; Tanaka & Miyajima, 2016).

The liver sinusoidal endothelial cells (LSECs) are highly specialized vasculature cells that create a selective permeable barrier due to the fenestrae; this contributes to the molecular exchange and product transformation in the liver (Poisson et al., 2017). The LSECs have a similar life span compared to those in large vessels, and they proliferate slowly but they maintain their characteristics for a longer time, although they can respond to the stimulus of growth factors such as vascular endothelial growth factor and fibroblast growth factor (Poisson et al., 2017). Furthermore, the LSECs have the ability to maintain the stellate cells out of a fibrogenesis state due to the control they have over the sinusoidal blood pressure.

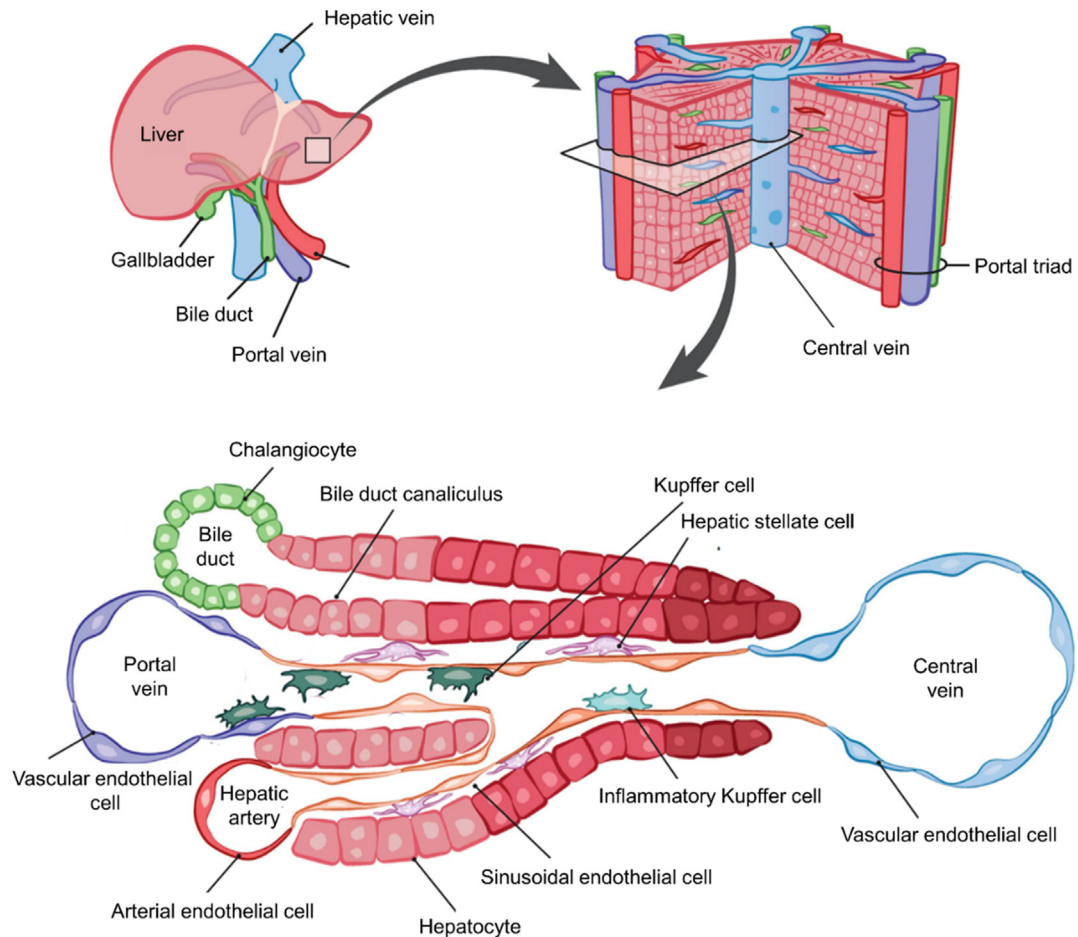
In between the hepatocytes and the sinusoidal endothelium, hepatic stellate cells (HSCs) are found in the Disse space and conform around 5%–8% of the total liver cells (Weiskirchen & Tacke, 2014). These are star-shaped cells characterized by the accumulation of retinoid (vitamin A) droplets from the diet and are crucial to its metabolism too. Stellate cells are also known to play a key role in inflammatory processes related to immunoregulation by allowing the infiltration of leukocytes to the liver, promote the differentiation of immune cells, stimulating lymphocyte production, or activating molecular pathways leading to apoptosis (Friedman, 2008). Moreover, HSCs are also considered key factors in the development of the fibrotic condition of the liver in the case of chronic injury; this will be further explored in the liver tissue damage section.

The Kupffer cells are resident macrophages of the liver that are adherent to LSECs, and therefore are located on the lumen of the sinusoids (Nguyen-Lefebvre & Horuzsko, 2015). They represent the first immune response mechanism to protect the organ against bacteria that can be present due to an infection, but mostly they are gastrointestinal bacterial debris and endotoxins that travel from the gut to the liver through the portal vein (Albillos, de Gottardi, & Rescigno, 2020). Since hepatocytes are the most abundant cells in liver tissue, they are often referred to as parenchymal cells; meanwhile sinusoidal endothelial, stellate, and Kupffer cells are known as nonparenchymal. The schematic representations of the liver and the single end of its lobule are shown in Figure 17.1 (MacParland et al., 2018).

Each cell type supports the homeostasis of the liver; therefore they all interact differently when a disequilibrium or a disease affects the organ and damages the tissue. Nowadays, there are several approaches taken into consideration to research the specific functions of the liver tissue when it is healthy or by inducing a specific condition. The importance of maintaining the phenotypical characteristics of the liver cells has been also correlated with keeping the native functions.

17.3 LIVER DISEASES

As previously mentioned, the liver's great capacity to detoxify the human body and to achieve the effective metabolism of fat, proteins, and drugs makes it highly prone to damage upon prolonged exposure to xenobiotics, pathogens, or products of an overly processed diet. Currently, liver diseases are the 12th most common cause of mortality worldwide and they involve a wide spectrum of pathologies that range from NAFLD to hepatocarcinoma (Heydari et al., 2020). Each disease activates a mechanism to help regenerate the damaged liver, but some may be counterproductive as the injury becomes more constant.

**FIGURE 17.1**

Schematic overview of the liver structure. The hepatic lobule is the building block of the liver that comprises portal triad (hepatic artery, the portal vein, and the bile duct), parenchymal cells (hepatocytes), and nonparenchymal cells (endothelial cells, cholangiocytes, Kupffer cells, and HSCs) aligned between a capillary network and a central vein. Blood enters the liver through the portal vein and the hepatic artery, flows through the sinusoids surrounded by sinusoidal endothelial cells, and empties into the central vein of each lobule. Bile secreted by hepatocytes is delivered into the bile ducts through the bile canaliculi. Kupffer cells are the resident macrophages in the liver and are present at the luminal side of the sinusoids, whereas the HSCs are located in close proximity to sinusoidal endothelial cells. *Reproduced under the Creative Commons Attribution License 4.0 for open access articles* ([MacParland et al., 2018](#)).

A common characteristic of most liver diseases is inflammation, which most likely will cause the tissue to become fibrotic; this means that there is an excess production of extracellular matrix (ECM) that is disruptive to the microarchitecture of the normal liver ([Tanaka & Miyajima, 2016](#)).

These changes also affect the microvilli of hepatocytes and cause the endothelial fenestrations to close, therefore creating a solute imbalance (Elpek, 2014). HSCs are constantly being studied due to their central role in the induction of fibrotic liver tissue after a chronic disease or an acute liver injury. The most known pathway that causes this effect is the transdifferentiating of HSCs to myofibroblasts with a high collagen production that intends to replace the damaged tissue (Weiskirchen & Tacke, 2014). However, the accumulation of the ECM can further activate pathways that maintain the fibrotic state. The production and inhibition of metalloproteinases and integrins that allow cytoskeleton communications lose their homeostatic state, so that the synthesis of ECM is continuous (Elpek, 2014). Additionally, other liver cell types change their functions in the state of injury and can contribute to fibrosis. For example, HSCs can engulf apoptotic hepatocytes and activate Kupffer cells that at the same time activate chemokine receptors (Elpek, 2014). The further study of the cellular mechanisms of fibrosis allows for new therapeutic key points to be targeted to possibly reverse the process.

A steatotic condition of the liver is described as the accumulation of excess fat in the liver and is commonly associated with NAFLD. It is considered NAFLD when the liver presents more than 5% of lipid storage and is one of the most common causes of chronic liver diseases (Di Ciaula et al., 2020). The reasons why the liver can accumulate lipids can vary from a metabolic syndrome, the use of medication that involves forced liver processing, endocrine dysfunction, or serious illnesses such as autoimmune or virus causing hepatitis (Marchisello et al., 2019). Steatosis is caused by an imbalance in the uptake and elimination of dietary lipids from the liver. The lipids are stored in dynamic organelles termed lipid droplets. They mostly store neutral lipids such as triglycerides or cholesterol esters but they can vary depending on the type of liver cell with this condition (Gluchowski, Becuwe, Walther, & Farese, 2017). These compounds are stored in the interior of the lipid droplets that also have a phospholipid monolayer surrounding it with specific binding proteins that can turn the cell into an emulsion ultimately interfering with the appropriate cell activities.

Cirrhosis is an advanced stage of fibrosis and is characterized by the distortion of the hepatic sinusoid. The hepatic sinusoid consists of fenestrated LSECs, forming a permeabilized wall on one side of perisinusoidal space (space of Disse). The LSECs possess vasoactive, inflammatory, and immune functions. The other side of the *perisinusoidal* space is lined with hepatocytes. Blood flow from both the hepatic portal vein and artery enters the hepatic sinusoids wherein oxygen and macromolecules diffuse across the LSEC to hepatocytes. HSCs present within perisinusoidal space coordinate with LSECs to regulate vascular tone by releasing vasoconstrictors such as cyclooxygenase 1 and thromboxane A₂, and vasodilators such as nitric oxide (Gracia-Sancho, Marrone, & Fernández-Iglesias, 2019). During cirrhosis, the *perisinusoidal* space is filled with scar tissue and LSECs become defenestrated (a process known as sinusoidal capillarization), preventing the proper oxygenation of hepatocytes. This causes impaired hepatocyte functions, intrahepatic resistance, and hepatocellular carcinoma (Schuppan & Afdhal, 2008).

The Kupffer cells are also activated by injurious factors such as alcohol or viral hepatitis *per se*; then, they directly attack hepatocytes and release harmful mediators. Sequentially, the platelet-derived growth factor receptor and transforming growth factor- β (TGF- β) are released following the activation of HSCs (Zhou, Zhang, & Qiao, 2014). Other important elements identified in cirrhotic tissue are the tumor necrosis factor family, profibrogenic interleukins (ILs), and microRNAs (miRNAs) that increase the expression of fibrosis-related

genes (Zhou et al., 2014). Oftentimes the disease is not detected on time and can be mortal depending on whether it is a compensated or a decompensated case (Mansour & McPherson, 2018). If the disease has covered the majority of the liver, then the only treatment is liver transplantation. This is a high burden for the healthcare systems due to the shortage of donors and the increasing number of cases. Therefore the necessity of bioengineered grafts or even organ replacement gives tissue engineering a complex challenge.

17.4 REGENERATION OF THE LIVER

The liver is the only solid organ with a high regenerative capacity after an injury that restores the hepatic volume and maintains the original liver-to-body weight ratio at 100%, which is critical for body homeostasis (Michalopoulos, 2017). Liver regeneration is a complex process and is categorized as acute or chronic regeneration depending on the type of injury that the liver tissue suffers. The understanding of the molecular mechanism of liver regeneration has been gained from the partial hepatectomy studies in animal models (Gilgenkrantz & Collin de l'Hortet, 2018). Unlike epithelial tissues such as the intestines and the skin wherein stem cells are responsible for cellular renewal and tissue homeostasis with high turnover, it has been assumed that all mature hepatocytes in the liver divide to maintain normal homeostasis and the turnover is low (Michalopoulos, 2017; Gilgenkrantz & Collin de l'Hortet, 2011). After 66% of the liver is removed, the uninjured liver activates more than a hundred genes including those encoding growth factor receptors, the most relevant being the epidermal growth factor receptor and the hepatocyte growth factor receptor (c-Met/HGFR). The heterogeneity of the hepatocyte functions depending on which zone they are located is also relevant to the process (Figure 17.2) (Matyas, Haskó, Liaudet, Trojnar, & Pacher, 2021). The hepatocytes in Zone 3 that are contiguous to the central vein express early liver progenitor markers such as *Tbx3* and *Axin2*, thus exhibiting higher proliferation (Saxena et al., 1999). Moreover, the Zone-1 hepatocytes that border the portal triad exhibit constant expression of progenitor markers besides also expressing bile duct-specific genes; therefore they are also referred to as hybrid hepatocytes. In contrast, if only one-third of the liver was removed, the hepatocytes would present initial hypertrophy to restore the liver mass with significantly fewer cell divisions (Gilgenkrantz & Collin de l'Hortet, 2018).

The repair mechanisms that activate regeneration may differ if the liver tissue is fighting a pathological condition, for instance, hepatitis, cirrhosis, or liver cancer. The hepatocytes that are found in the center of the lobule can process xenobiotics through cytochrome P450, and this same reaction liberates reactive oxygen species that can trigger localized necrosis in an acute injury (Tanaka & Miyajima, 2016). The induced inflammation also accompanies the proliferation of new hepatocytes for 72 hours aided by nonparenchymal cells. The inflammatory stimulus on the HSCs not only triggers the secretion of hepatocyte growth factor (HGF) but also activates the production of collagen to provide replacement for the mechanical support of the damaged tissue (Tanaka & Miyajima, 2016). At the same time, the LSECs allow the entrance of leukocytes to the affected area and secrete HGF. If the tissue damage remains constant, the inflammation can induce fibrosis that can ultimately lead to cirrhosis or hepatocellular carcinoma. Furthermore, chronic inflammation contributes to an increase in liver progenitor cells (LPCs) that can aid to regenerate the tissue

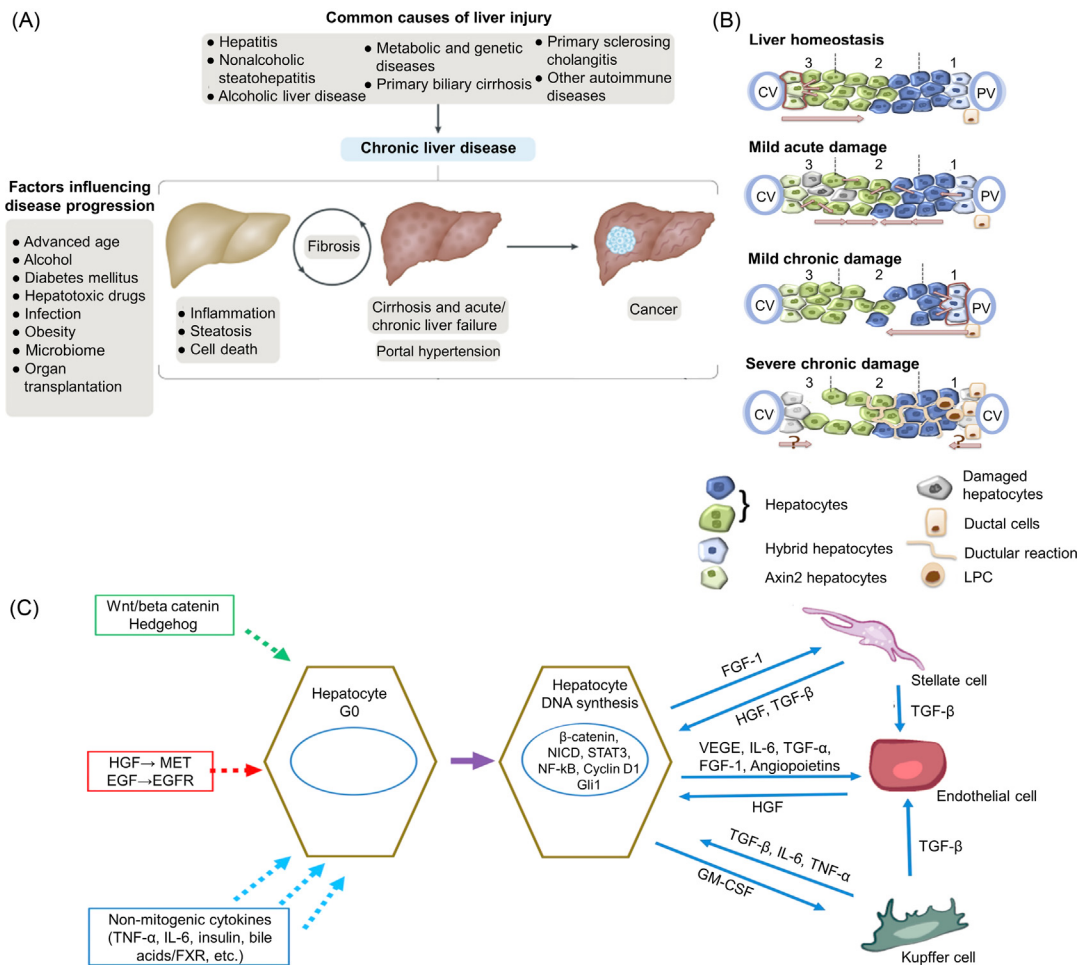


FIGURE 17.2

Liver injury and regeneration. (A) Several mechanisms that contribute to hepatocyte damage and the liver function deterioration include drug-induced toxicity, alcoholic and NAFLD, hepatotropic infections, and hereditary genetic disorders. Fatty liver diseases, both alcoholic fatty liver disease and NAFLD, are caused by the chronic accumulation of fat droplets in the liver. Liver cirrhosis can be caused by hepatitis B and C virus infections, chronic alcoholism, chronic inflammation, chronic fat accumulation, and autoimmune diseases such as *primary biliary cholangitis*. Cirrhosis is typically characterized by structural changes in the hepatic sinusoid and the overaccumulation of ECM known as hepatic scar. These changes result in the deterioration of liver functions that increase the risk of hepatic failure and hepatocellular carcinoma. *Reproduced with permission from Springer Nature (Matyas, Haskó, Liaudet, Trojnar, & Pacher, 2021).* (B) Cells involved in liver regeneration depend on the type of injury. Axin-2-positive pericentral vein hepatocytes (pale green) exhibit a higher proliferative capacity than the hepatocytes in the intermediate (green and blue) or periportal (pale blue) regions during liver homeostasis. After a mild acute injury, all undamaged hepatocytes undergo synchronized proliferation until the original liver mass is restored. Hybrid periportal hepatocytes (pale blue), possessing phenotypes of both hepatocytes and biliary epithelial cells, preferentially proliferate and restore the liver after mild chronic hepatic damage. Ductular reaction, a reactive lesion characterized by the proliferation of intrahepatic bile ducts, is induced by severe chronic liver damage. The ductular reaction is

(Continued)

due to their differentiation capacity to turn to hepatocytes and biliary epithelial cells; however, their action mechanism is still being elucidated. The constant activation of abnormal cell mechanisms that require constant proliferation can also have negative consequences like neoplasia; and therefore it is relevant to understand the activated pathways to consider the implications of a bioengineered graft (Michalopoulos & Bhushan, 2021).

The regenerative capacities of the liver are constantly studied given its relationship with crucial functions. It is known that the liver regenerates to achieve homeostasis in relation to the body's prolonged state, for example, in pregnancy or severe weight loss. However, in a normal state, the cell turnover can be slower than needed to regenerate after an acute injury or a chronic disease (Michalopoulos & Bhushan, 2021). Alternate paths to aid liver regeneration, including engineering functional liver tissue constructs *via* various strategies such as 3D bioprinting, are being researched to provide a therapeutic option for patients with severe liver tissue damages.

17.5 LIVER TISSUE ENGINEERING AND 3D BIOPRINTING

As previously explored in this chapter, liver diseases can lead to the necessity of organ transplantation or partial removal. Even though this specific organ has a higher regenerative capacity than many other organs, the correct molecular cues should be activated for it to develop properly. The tissue engineering field is dedicated to achieving substitutes that can maintain functions or trigger regeneration in a damaged or a lost organ (Poomathi et al., 2020). As such, the major goals of tissue engineering are to mimic the original structure of the tissue, to reduce immune response when grafting the construct, and to achieve the proper mechanical and degradation properties of the scaffolding to allow the new cells to integrate into the native tissue. During the early 2000s, 3D printing catalyzed itself as a useful tool for the medical field. Even if its initial intended purpose was to develop prosthesis and dental implants, it was soon realized that these principles could be combined with tissue engineering to create scaffolds that allow cell growth (Poomathi et al., 2020). In recent years, with the advances in cell biology, materials science, and engineering, 3D bioprinting technologies have been widely adopted for tissue engineering and other biomedical applications (Xie, Gao, Lobo, & Webster, 2020). 3D bioprinting utilizes highly tunable material designs to achieve rapid assembly of tissue constructs *in vitro* potentially at scale (Maharjan, Alva, et al., 2021; Ozbolat, Peng, & Ozbolat, 2016; Zhang et al., 2017).

◀ associated with the activation and proliferation of hepatic progenitor cells (LPCs), which differentiate into *hepatocytes* or *cholangiocytes* leading to liver tissue repair. Zone 1: periportal region, Zone 2: intermediate region, and Zone 3: centrilobular region. CV, central vein; PV, portal vein. *Reproduced with permission from Elsevier (Gilgenkrantz & Collin de l'Hortet, 2018)*. Schematic overview of the growth-regulatory signaling interactions between hepatocytes and nonparenchymal liver cells (stellate, endothelial, and Kupffer cell) during the liver regeneration. *Reproduced with permission from Wiley adopted from (Michalopoulos, 2017)*.

17.5.1 BIOINKS FOR 3D BIOPRINTING OF LIVER TISSUES

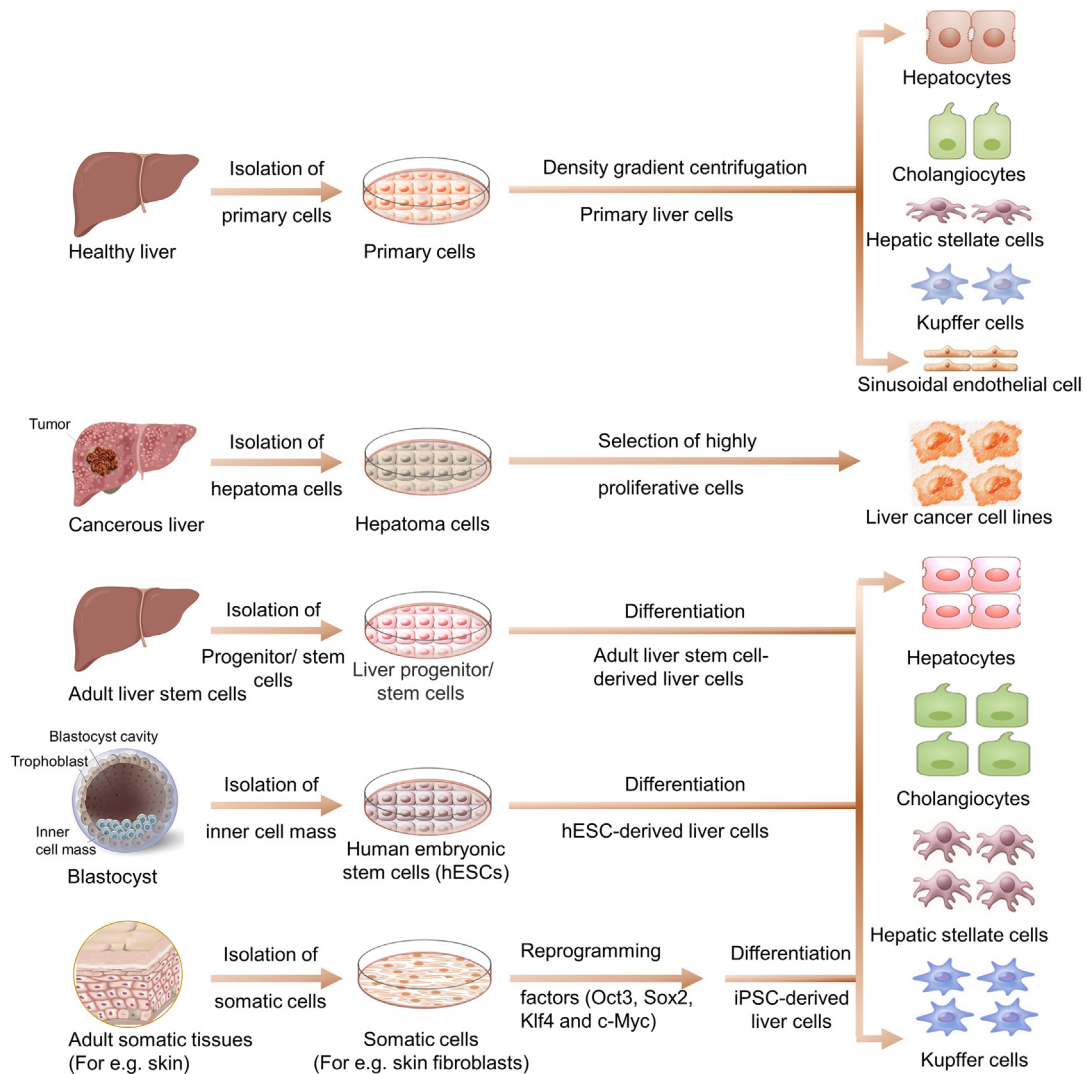
The biomaterial choice when biofabricating a specific tissue is very relevant as a meticulously designed composition could favor the stability of a specific type of cells more than others. These need to be relevant and compatible with the tissue of interest. The cells and the surrounding hydrogel oftentimes constitute the bioink. The liver comprises liver parenchymal cells, mainly hepatocytes, and nonparenchymal cells including LSECs, HSCs, and Kupffer cells. These hepatic cells have their specific functions in the liver as discussed in the earlier section. Primary human liver cells are considered to be the gold standard for the engineering of human-relevant liver models *in vitro* (Kim et al., 2018). However, due to the lack of the constant source of primary human liver cells, their wide applications in *in vitro* liver tissue engineering are limited. In addition, primary human liver cells are normally difficult to expand and maintain *in vitro* (Zeilinger, Freyer, Damm, Seehofer, & Knöspel, 2016). Therefore liver cancer cell lines, mostly HepG2 and HepG2-C3A cells (Maharjan, Alva, et al., 2021; Maharjan, Bonilla, et al., 2021) as well as human embryonic stem cell (hESC)-derived liver cells and human-induced pluripotent stem cell (hiPSC)-derived liver cells (Goulart et al., 2019; Ma et al., 2016), have been widely used in 3D bioprinting of liver tissues (Figure 17.3).

For the liver, the native components of the ECM need to be taken into consideration to attain growth and cell signaling. It is known that this specific ECM is rich in collagen and glycosaminoglycans, which also provide mechanical support (Zhang et al., 2017). The most commonly used hydrogels for liver tissue bioprinting are alginate, collagen, gelatin and its derivatives, and decellularized ECM (dECM). Alginate, a seaweed extract, is oftentimes used in 3D bioprinting of liver tissue due to its biocompatibility, excellent printability, and low cost (Jeon et al., 2017; Maharjan, Alva, et al., 2021; Wu, Lin, Wenger, Tam, & Tang, 2018). Similarly, gelatin and its photocurable derivatives such as gelatin methacryloyl (GelMA) are frequently adopted for liver 3D bioprinting (Ma et al., 2016; Maharjan, Bonilla, et al., 2021; Wu, Wenger, Golzar, & Tang, 2020). The GelMA hydrogel is biocompatible and can be tuned to achieving desirable mechanical properties relevant to those of the human hepatic tissue (Ying, Jiang, Yu, & Zhang, 2018).

Collagen, the major component of the liver ECM, represents the primary choice for developing 3D-bioprinted liver tissues. However, because of their poor mechanical strength, bioprinted collagen scaffolds are *easy to collapse* or deform (Osidak, Kozhukhov, Osidak, & Domogatsky, 2020). Thus, the collagen hydrogel is usually combined with other materials such as polycaprolactone (Shim, Kim, Park, Park, & Cho, 2011) or hyaluronic acid (Mazzocchi, Devarasetty, Huntwork, Soker, & Skardal, 2018) to improve the *mechanical strength* of the collagen-based hydrogels. In addition, it is also a considerable challenge that the cells have the possibility to rearrange and conform to the structural organization *as the native liver tissue*. Also, when formulating a bioink with hydrogels, the concentration of each component will have a secondary effect on the viscosity and printing parameters, thus ultimately affecting cell viability and functions (Nguyen-Lefebvre & Horuzsko, 2015).

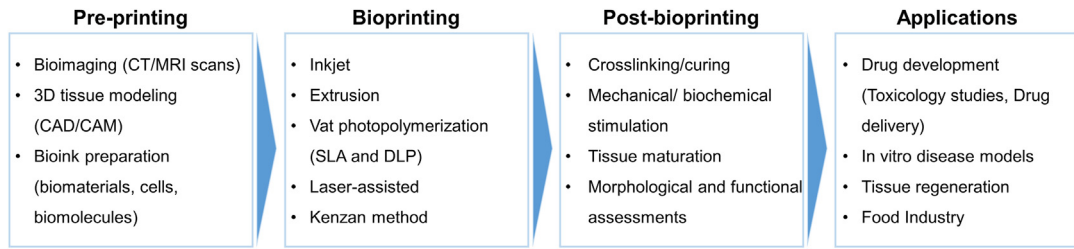
17.5.2 3D BIOPRINTING

3D bioprinting is a powerful tool that typically involves layer-by-layer placement of living cells, ECM-like hydrogels, or/and other biologics in spatially predefined patterns to create highly organized functional tissues or organs (Heinrich et al., 2019; Moroni et al., 2018; Murphy & Atala, 2014; Ozbolat et al., 2016; Zhang et al., 2017). The important feature of bioprinting is the ability to precisely control structural and biomaterial properties in 3D to achieve the key architectural features and functionality of complex tissues.

**FIGURE 17.3**

Sources of major hepatic cells for 3D bioprinting of human liver tissues. Normal primary liver cells including primary hepatocytes, cholangiocytes, HSCs, Kupffer cells, and LSECs are derived from a healthy liver. Hepatic cancer cell lines, mostly hepatocyte cell lines, are derived from liver tumors. Other sources of liver cells include adult liver stem cell-derived liver cells, hESC-derived liver cells, and iPSC-derived liver cells. Part of the figure generated using BioRender.

The bioprinting process is usually accomplished in multiple steps that include computed-tomography or magnetic resonance imaging of the desired tissue, and subsequent use of computer-aided design/computer-aided manufacturing (CAD/CAM) software to obtain the tomographic and architectural information

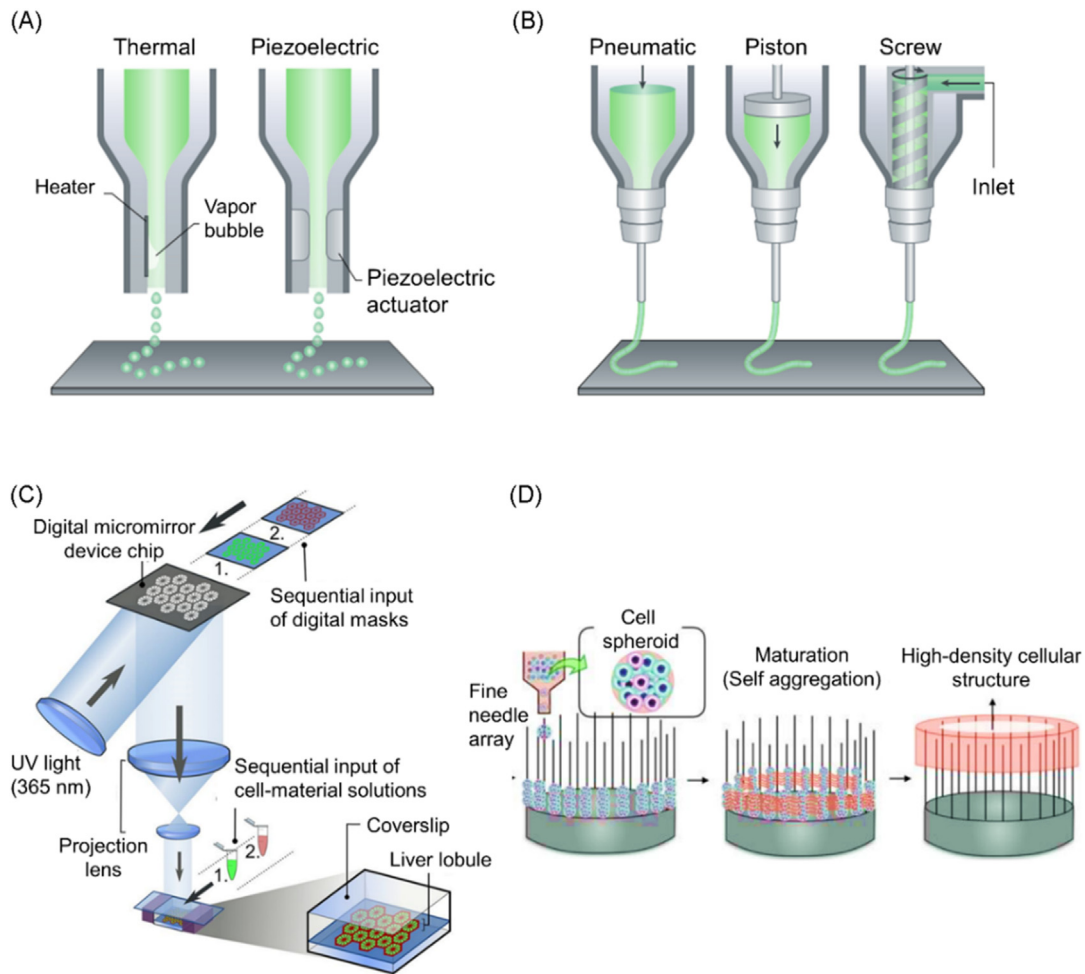
**FIGURE 17.4**

Overview of steps involved in 3D bioprinting of (liver) tissue constructs and their applications in different fields.

of the tissue (Murphy & Atala, 2014; Zhang et al., 2017), selection of physiologically relevant biomaterials and cells, and bioprinting of the tissue construct followed by tissue maturation *in vitro*. The overview of the steps involved in a general 3D bioprinting process is shown in Figure 17.4 (Bishop et al., 2017). The main strategies of 3D bioprinting include biomimicry that aims to emulate the structural and functional features of tissue or organ (Reed, Klumb, Koobatian, & Viney, 2009), autonomous biological self-assembly that uses embryonic organ development as a guide for cellular organization and tissue fusion (Ingber et al., 2006), as well as the use of mini-tissue building blocks that can assemble into the larger structures by biologically inspired rational design, self-assembly, or a combination of both (Mironov et al., 2009; Murphy & Atala, 2014; Yu et al., 2016). The integration of these different 3D bioprinting strategies is often required to generate complex tissue structures with relevant functional, structural, and mechanical properties. Inkjet-based bioprinting (Arai et al., 2017; Cui, Boland, D'Lima, & Lotz, 2012; Matsusaki, Sakaue, Kadowaki, & Akashi, 2013; Xu et al., 2013), extrusion-based bioprinting (Bhise et al., 2016; Faulkner-Jones et al., 2015; Kim et al., 2017; Lee et al., 2017; Nguyen et al., 2016; Wang et al., 2006), and vat photopolymerization-based bioprinting (Ma et al., 2018; Neiman et al., 2015; Zhang, Hu, Wang, Tao, & Gou, 2019) are the most commonly adopted bioprinting techniques for the generation of biomimetic tissues. Moreover, the spheroid-based bioprinting method, known as the Kenzan method, has been developed to create tissue constructs including the liver tissue (Yanagi et al., 2017).

17.5.2.1 Inkjet-Based Bioprinting

Inkjet-based bioprinting, also known as drop-on-demand bioprinting, allows the precise positioning of individual droplets of the bioink onto a substrate using thermal (Cui et al., 2012) or piezoelectric (Saunders, Gough, & Derby, 2008) methods (Figure 17.5A). Thermal inkjet bioprinting involves electrical heating of the printhead up to 200°C–300°C for a few microseconds to generate pressure pulses that eject bioink droplets from the nozzle (Cui et al., 2012; Gao, Yonezawa, Hubbell, Dai, & Cui, 2015). High speed, high resolution, and low cost are the advantages of thermal inkjet bioprinting. However, the limitations of this approach include potential damage of the cells due to high temperature and mechanical stress, nonuniform droplet sizes, and frequent clogging of the printhead (Gudapati, Dey, & Ozbolat, 2016). Piezoelectric inkjet bioprinting uses a piezoelectric actuator to generate acoustic waves inside the printhead that form bioink droplets at regular intervals. When a voltage is applied, the piezoelectric material changes its shape rapidly and generates pressures required to form and eject bioink droplets from the printhead (Saunders et al., 2008; Wijshoff, 2010). This bioprinting method requires no heat, thus preventing damage of cells due to heat, and it

**FIGURE 17.5**

3D bioprinting techniques. (A) Inkjet-based bioprinting. *Reproduced with permission from Springer Nature*, (B) Extrusion-based bioprinting systems. *Reproduced with permission from Springer Nature (Moroni et al., 2018)*, (C) Vat photopolymerization-based bioprinting. *Reproduced under the Creative Commons Attribution License 4.0 for open access articles (Ma et al., 2016)*, and (D) The Kenzan bioprinting. *Reproduced under the Creative Commons Attribution License 4.0 for open access articles (Jeong, Nam, Jang, & Lee, 2020)*.

generates uniform droplets (Saunders et al., 2008). The common limitation of inkjet-based bioprinting is that the viscosity of the bioink should be low roughly 10 mPa s in order to form droplets because excessive force is required to eject droplets of higher viscosity bioinks (Kim, Choi, Kim, Chan Choi, & Cho, 2010). Inkjet-based bioprinting has been employed to develop liver tissues (Arai et al., 2017; Parsa, Gupta, Loizeau, & Cheung, 2010).

17.5.2.2 Extrusion-Based Bioprinting

Extrusion-based bioprinting is arguably the most common 3D bioprinting approach and involves the continuous extrusion of a bioink through a nozzle using either pneumatic pressure or mechanical dispensing systems (Maharjan, Alva, et al., 2021; Ozbolat & Hospodiuk, 2016; Pi et al., 2018). Extrusion bioprinters consist of a bioink dispensing system and stage that are capable of movement along the x , y , and z axes facilitating the precise layer-by-layer patterning of bioink to fabricate complex structures based on CAD design (Figure 17.5B). Extrusion-based bioprinting is compatible for controlled deposition of bioinks with a wide range of viscosities ranging from 30 mPa s to $>6 \times 10^7$ mPa s (Chang, Boland, Williams, & Hoying, 2011). Bioinks composed of shear-thinning biomaterials are commonly used for extrusion-based bioprinting (Liu et al., 2017). Another advantage of extrusion-based bioprinting is the ability to bioprinting high-cell density bioinks (Diamantides, Dugopolski, Blahut, Kennedy, & Bonassar, 2019) or even cell-only bioinks including cell spheroids to create physiological relevant 3D tissue constructs with high-cell densities (Daly, Davidson, & Burdick, 2021; Jeon et al., 2019; Norotte, Marga, Niklason, & Forgacs, 2009). Relatively low cell viability (approximately 40%–86%) primarily due to shear stress and dispensing pressure (Chang, Nam, & Sun, 2008), as well as insufficient bioprinting resolution and speed (Murphy & Atala, 2014), is the major limitations of extrusion-based bioprinting. Extrusion-based bioprinting has been used to fabricate multiple tissue types (Cao et al., 2019; Maharjan, Alva, et al., 2021; Pi et al., 2018; Ying, Jiang, Maharjan, et al., 2018; Ying et al., 2020) including liver tissue constructs (Bhise et al., 2016; Faulkner-Jones et al., 2015; Hiller et al., 2018; Lee et al., 2017; Wang et al., 2006), which will be discussed in detail later.

17.5.2.3 Vat Photopolymerization-Based Bioprinting

Vat photopolymerization-based bioprinting is an emerging nozzle-free bioprinting technique that involves layer-by-layer bioprinting of the photocurable bioink placed in a reservoir to generate 3D tissue constructs (Figure 17.5C) (Li et al., 2020). Planar serial cross-sectional images obtained by slicing of the 3D CAD model are projected onto the surface of the photocurable bioink in a layer-by-layer fashion and the uncured bioink is finally washed away to obtain a 3D tissue construct. Stereolithography apparatus (SLA) and digital light processing (DLP) are two common *vat* photopolymerization-based bioprinting technologies. While SLA typically uses an ultraviolet (UV) laser beam with point scans, DLP harnesses UV or visible light from a digital micromirror device or liquid crystal display to project two-dimensional (2D) images. While both *vat* photopolymerization-based bioprinting modalities offer advantages such as high resolution and the ability to fabricate highly complex 3D tissue constructs as compared to other bioprinting methods (Kumar & Kim, 2020; Li et al., 2020), DLP bioprinting is generally faster when compared to SLA (Cui, Nowicki, Fisher, & Zhang, 2017). Nevertheless, each of these methods is constantly being updated to improve the bioprinting resolution and speed. Complex microstructures of hepatic lobules that have been constructed using *vat* photopolymerization-based bioprinting (Ma et al., 2016, 2018, 2020) will be discussed later in detail.

17.5.2.4 Kenzan Bioprinting

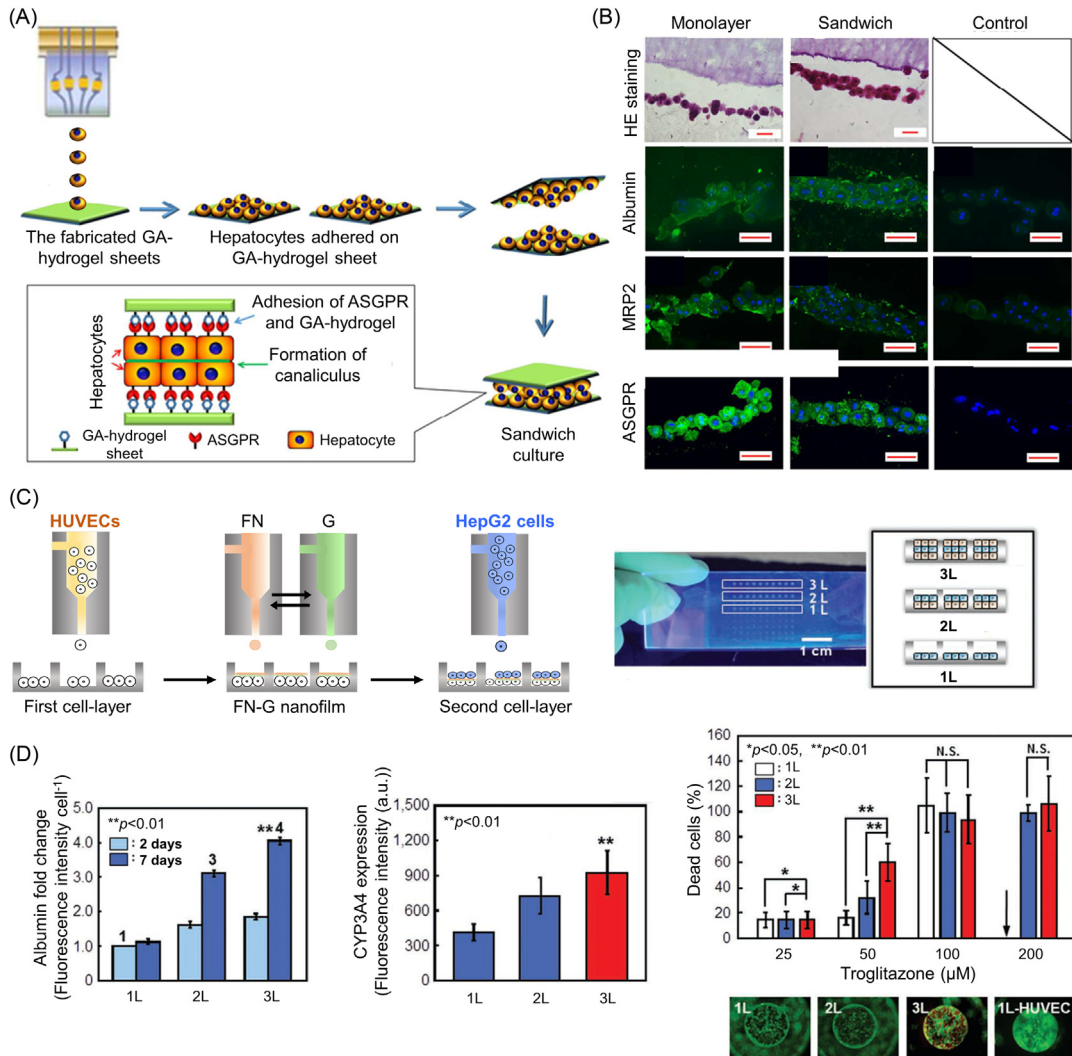
The Kenzan method is a cutting-edge approach that bioprints cell spheroids individually into a microneedle array, where the physical closeness of the spheroids causes them to fuse together and

achieve a higher level of survivance (Figure 17.5D) (Murata, Arai, & Nakayama, 2020; Yanagi et al., 2017; Jeong, Nam, Jang, & Lee, 2020). This method earlier on involved the development of a special bioprinter termed the Bio-3D printer by Regenova that includes the use of cameras to capture and confirm the positions of the needles and the spheroids. The key component of this system is the microneedle array that gives the name to this technique “the Kenzan.” This method does not use hydrogel biomaterials but only multicellular spheroids prepared by seeding cells in a nonadhesive well plate with round bottoms that facilitate the creation of the cell spheroids of approximately 600 μm in diameter (Murata et al., 2020). After the spheroids are robotically positioned in a microneedle array in an overlaid manner, they are cultivated within the microneedle array for several days to allow them to fuse together to form a 3D tissue structure. Finally, the fused 3D tissue construct is released from the Kenzan by sliding an already installed plate that would liberate it from the device, so it can be further cultivated and studied for further maturation and downstream studies. Even though mostly tubular constructs have been developed with this capacity, the positioning precision that the Kenzan has could open a new possibility for liver bioprinting that allows spheroids from parenchymal and nonparenchymal cells to be placed in an order that is close to the native structure while achieving high cellular densities that is physiologically relevant.

17.6 3D-BIOPRINTED LIVER TISSUES

As mentioned above, the complex microarchitecture of the liver and the heterogeneous mixture of cells spatially arranged in a specific order currently cannot be achieved in 2D culture systems. Currently, the biomimetic resemblance is one of the key points that is aimed to achieve with a 3D construct, due to the necessity to keep the hepatic cells in their highly metabolic functional state. To this end, 3D bioprinting provides the possibility to provide the cells with physiologically relevant structural support that can maintain the phenotypical characteristics and stimulate intercellular communications within an ECM-emulating biomaterial and configuration (Ma et al., 2020). This can allow to preserve the crucial features of the functional liver cells, for example, the polarity aspect of hepatocytes. Nevertheless, various strategies are constantly being explored to achieve optimal bioprinting and postbioprinting culture targeting to create constructs that can possibly be transplanted into a patient (Kryou, Leva, Chatzipetrou, & Zergioti, 2019).

A wide range of liver tissues with different levels of complexities have been developed by employing various bioprinting methods. In one of the earlier studies, the human liver cancer cell lines, HepG2 cells, were dispensed onto a collagen hydrogel (1.5 mg/mL) by piezoelectric inkjet bioprinting and a cell survival rate of >95% after 2 days of bioprinting was shown (Parsa et al., 2010). The addition of 0.05% pluronic, a biocompatible surfactant, facilitated the droplet formation through a piezoelectric nozzle. In another study, piezoelectric inkjet bioprinting was used for generating a liver scaffold wherein two layers of primary mouse hepatocytes were sandwiched in between galactosylated alginate (GA)-hydrogel sheets (Arai et al., 2017). The GA-hydrogel sheet was fabricated by adding 1.2% GA solution into a polydimethylsiloxane (PDMS) mold containing 20 mg/mL of barium chloride solution. The GA-hydrogel was able to promote adhesion of hepatocytes and regulate hepatocyte cell polarity due to the interaction between the galactose chain of GA-hydrogel and asialoglycoprotein-receptor (ASGPR) of hepatocytes (Figure 17.6A). The

**FIGURE 17.6**

3D liver constructs fabricated by inkjet-based bioprinting. (A) Schematics of the bioprinting of primary mouse hepatocyte layers on the GA-hydrogel sheets and generation of the sandwich culture system. (B) HE staining and immunocytochemical staining of hepatocytes for albumin, MRP2, and ASGPR in monolayer and sandwich culture systems after 2 days of culture. *Reproduced with permission from Wiley (Arai et al., 2017).* (C) Schematics illustrating the fabrication of 3D human liver microtissue constructs by layer-by-layer bioprinting of HepG2 cells or HUVECs and fibronectin-gelatin nanofilms followed by the photograph of 440 microarrays of 3D-liver tissue constructs with 1 L (HepG2 monolayer), 2 L (HUVEC/HepG2), and 3 L (HUVEC/HepG2/HUVEC) of cells under UV light. (D) Albumin secreted by HepG2 cells 2 or 7 days of culture, CYP3A4 expressed by HepG2 cells 1 day of culture, and dose-dependent cytotoxicity of troglitazone in coculture after 2 days of culture. *Reproduced with permission from Wiley adopted from (Matsusaki et al., 2013).*

bioprinted hepatocytes in this 3D sandwich culture system maintained high-cell viability and expressed liver-specific markers such as albumin, multidrug resistance protein 2 (MRP2), and ASGPR within 2 days of the culture period, demonstrating the functionality of the bioprinted hepatocytes (Figure 17.6B). Similarly, by using piezoelectronic inkjet bioprinting, a microarray of 440 human liver microtissue constructs were fabricated by layer-by-layer bioprinting of HepG2 cells or human umbilical vein endothelial cells (HUVECs), and fibronectin-gelatin (FN-G, 0.2 mg/mL) films in a microwell plastic plate (Figure 17.6C) (Matsusaki et al., 2013). These 3D liver microtissue constructs were cultured for up to 7 days and evaluated for liver functions as well as dose-dependent cytotoxicity of troglitazone. The liver functions such as albumin secretion, cytochrome P450 (CYP450) secretion, and CYP450 metabolic activity were shown to be improved in three-layered 3D human liver microtissue constructs with alternating HepG2 and HUVECs layers as compared to single- and double-layered constructs (Figure 17.6D).

Similarly, extrusion-based bioprinting has been used widely to generate liver tissue constructs. In a study by Lee et al., grid-patterned hepatic scaffolds were fabricated by extrusion bioprinting of human adipose-derived stem cell (hADSC)- and hADSC-derived hepatocyte-like cell (AHLIC)-encapsulated collagen bioink (Lee et al., 2017). These bioprinted hepatic scaffolds were crosslinked with 0.25-mM genipin to achieve the mechanical strength of the construct appropriate for *in vivo* transplantation. The scaffolds were transplanted into the right lobe of the rat liver and after 4 weeks of transplantation, it was found that hADSCs and AHLICs within the hepatic scaffolds migrated into the portal veins of the hepatic lobules as shown by immunohistochemical staining of cells with a human nucleus-specific marker. Furthermore, these hASC- and AHLIC-laden scaffolds were transplanted in rats with fibrotic liver induced by dimethylnitrosamine, a potent hepatotoxin. The rats with a fibrotic liver that were transplanted with scaffolds possessing only hADSCs and untransplanted rats were used as control groups. After a week of transplantation, serum levels of aspartic aminotransferase, alanine aminotransferase, total bilirubin, and alkaline phosphatase in hADSC- and AHLIC-laden scaffold-transplanted rats were found to be at the similar level to those in the untransplanted group, whereas the serum levels of these proteins decreased in the hADSC scaffold-transplanted group. These results suggested that hADSC-laden hepatic scaffolds may facilitate the regeneration of damaged liver tissue.

Hiller et al. presented extrusion bioprinting of HepaRG liver cell-laden composite bioink composed of alginate (2%), gelatin (3%), and human lung-derived dECM (1 mg/mL) to obtain grid-patterned liver tissue constructs (Figure 17.7A) (Hiller et al., 2018). The viability of HepaRG cells within bioprinted scaffolds was found to be high, as shown by cell viability assays at day 1 and day 7, and they exhibited high metabolic activities, as demonstrated in tetrazolium hydroxide salt assay, lactate dehydrogenase (LDH) assay, albumin secretion, and CYP450 3A4 (CYP3A4) activity (Figure 17.7B). These liver constructs were transduced with adeno-associated virus (AAV.6) vector which mediated knockdown of an average of 70%–80% cyclophilin B (hCycB), an endogenous target to study silencing efficiency *via* RNA interference. Transduction of the liver constructs with AAV2.6 mediated approximately 70%–80% hCycB knockdown (Figure 17.7C). Furthermore, the bioprinted liver tissue constructs were infected with human adenovirus 5 and it was shown that the infectious adenoviral particles were increased between day 3 and day 7 postinfection (Figure 17.7D), suggesting that 3D-bioprinted liver constructs could be used to study virus biology and antiviral drug testing. Similarly, HepG2 cell-encapsulated alginate (3%) (Jeon et al., 2017), mouse primary hepatocyte-encapsulated alginate (3%) (Kim et al., 2017), HepG2 cell-encapsulated

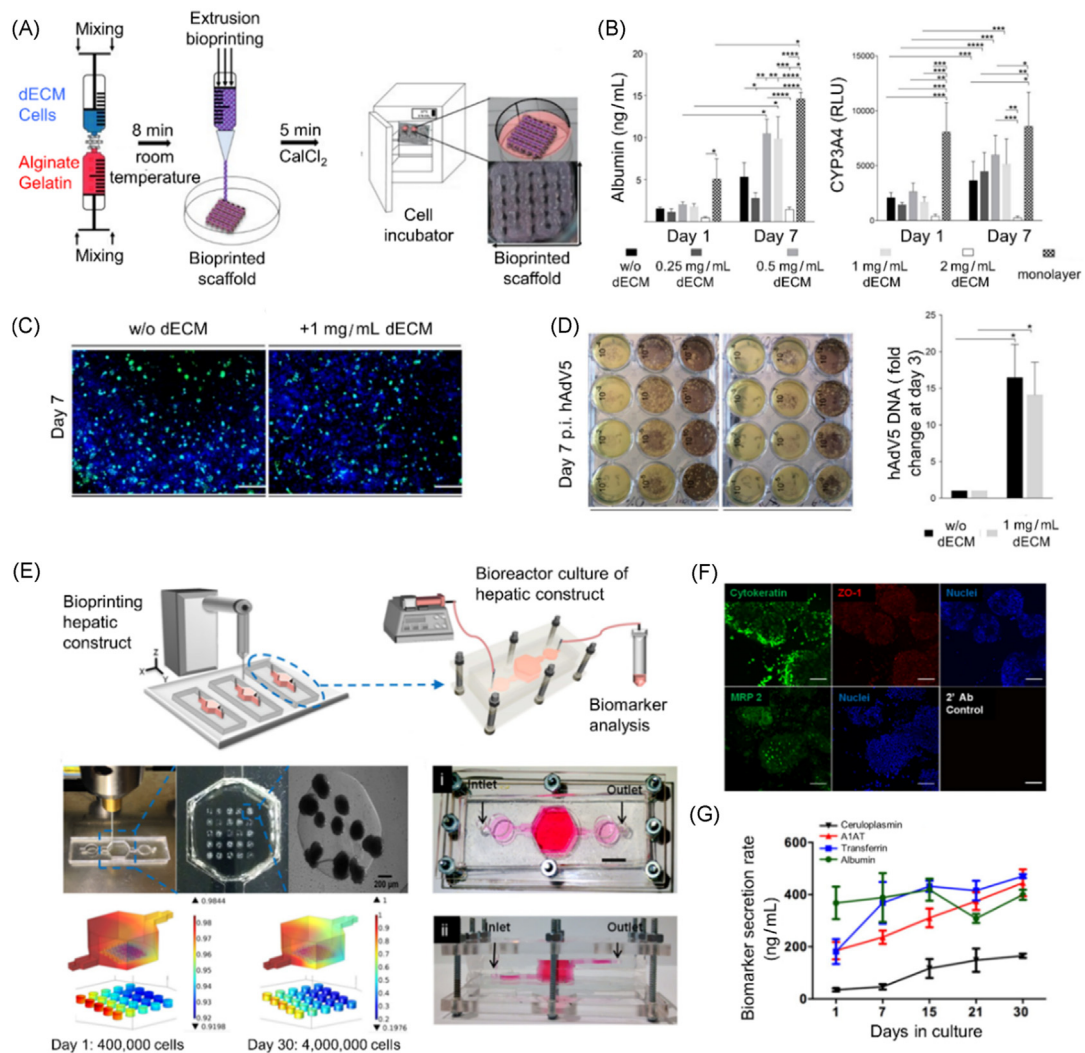


FIGURE 17.7

3D liver constructs fabricated by extrusion-based bioprinting. (A) Schematics of extrusion bioprinting of HepaRG cell-laden hybrid alginate/gelatin/human lung-derived dECM to fabricate grid-patterned liver tissue constructs. (B) Albumin secretion and CYP3A4 activity of bioprinted mature HepaRG cells within tissue construct. (C) Fluorescence images showing the AAV2.6 vector transduction and distribution within mature HepaRG cell-laden liver tissue constructs in the presence and absence of 1 mg/mL of dECM at day 7. (D) Adenovirus infection and adenoviral DNA replication within the mature HepaRG cell-laden tissue constructs, determined by a quantitative polymerase chain reaction in the presence and absence of 1 mg/mL of dECM at day 7. Reproduced under the Creative Commons Attribution License 4.0 for open access articles (Hiller et al., 2018). (E) Schematic and real images of the hepatic bioreactor culture platform integrated with a bioprinter and biomarker analysis system along with the simulation of an oxygen concentration gradient in the bioreactor. (F) Functionality of hepatic spheroids cultured in the bioreactor as shown by confocal images of spheroids immunostained for cytokeratin 18, ZO-1 tight junction binding protein, and MRP2 biliary canalicular transporter after 30 days of bioprinting. (G) The rate of secretion of hepatic biomarkers including albumin, transferrin, A1AT, and ceruloplasmin, by HepG2/C3A spheroids cultured in the bioreactor for 30 days. Reproduced with permission from IOP publishing (Bhise et al., 2016).

GelMA (10%–20%) (Billiet, Gevaert, De Schryver, Cornelissen, & Dubruel, 2014), and HepG2 cell-encapsulated liver-derived dECM (1.5% and 3%) (Lee et al., 2017) were bioprinted by the extrusion bioprinting approach to obtain grid-patterned liver constructs. These studies were mainly focused on the development of bioinks for 3D bioprinting of liver tissue constructs, in which rheological properties of the bioink, bioprinting parameters as well as cytotoxicity and functionality of the cells were studied. Furthermore, Huh7 cell-encapsulated gelatin (10%) was bioprinted to obtain scaffolds with grids at different angles to study the effect of the pore size and the angle between the grid lines on the viability and liver-specific functions of the cells such as albumin secretion, CYP activity, and bile transport (Lewis, Green, & Shah, 2018).

In another study by Nguyen et al., disk-shaped liver tissue constructs were created, in which 100% cellular paste of patient-derived primary human hepatocytes was deposited in the center whereas HUVEC- and HSC-encapsulated NovoGel hydrogel was deposited in the surroundings onto the membranes of 24-well Transwell culture inserts (0.4 μ m). These disk-shaped liver tissue constructs were used to study drug-induced liver injury (DILI) (Nguyen et al., 2016). These liver tissue constructs were treated with hepatotoxicant trovafloxacin to induce DILI and compared to that with levofloxacin. The results revealed that trovafloxacin induced significant toxicity in liver tissue constructs at clinically relevant doses ($\leq 4 \mu$ M) in a dose-dependent manner. Later, Norona et al. used these 3D-bioprinted liver tissue constructs to examine methotrexate (MTX)- and thioacetamide (TAA)-induced fibrogenesis (Norona, Nguyen, Gerber, Presnell, & LeCluyse, 2016). The same liver tissue constructs were used to analyze the effects of TGF- β 1 exposure and MTX for 28 days as well as evaluated the role of KCs on liver injury and fibrogenesis in a separate study (Norona et al., 2019). Recently, a highly vascularized hepatic lobule structure was fabricated by extrusion bioprinting of a high-cell density bioink comprising HepG2-C3A and EA.hy 926 endothelial cells embedded in neutralized collagen (3%) (Kang et al., 2020). The cell-free alginate (3%) was used as the sacrificial hydrogel to create a lumen and with a central vein in the fabricated hepatic lobule. While HepG2-C3A cells resided within the hepatic lobes, the EA.hy 926 endothelial cells formed the exterior covering, an endothelium lining of the lumen, and interconnected the exterior and the lumen of the well-organized lobular construct. The structural integrity and functionality of the bioprinted hepatic lobules were demonstrated by the immunostaining of constructs with CD31 for endothelial cells, and albumin and MRP2 expressions for HepG2-C3A cells. Furthermore, secretions of albumin and urea by HepG2-C3A cells within these lobular constructs were found higher as compared to a nonengineered group.

In addition to the above studies, extrusion bioprinting has also been used to generate liver organoids. Yang et al. bioprinted 3D liver organoids using bioink comprising HepaRG cells, gelatin (5%), and alginate (1%) (Yang et al., 2021). These liver organoids were named hepatorganoids. Cholangiocytic differentiation of the hepatorganoids was induced with dimethyl sulfoxide (DMSO) at different concentrations (0.5%, 1%, and 2%), where it was found that with increasing concentration of DMSO, the cholangiocytic differentiation of HepaRG was enhanced as revealed by the increased number of cytokeratin 19 (CK19)-positive cells and reduced albumin-positive cells in hepatorganoids. Albumin-positive cells were most abundant at 0.5%-DMSO induction. Similarly, secretion of albumin, alpha-1 antitrypsin (A1AT), factor VII, and factor IX were significantly increased at 0.5%-DMSO induction as compared with those in undifferentiated HepaRG cells. These hepatorganoids were also shown to acquire CYP1A2 and CYP3A4 activities. The hepatorganoids were then transplanted into abdominal cavities of mice with chronic liver injury. The mice that received hepatorganoids showed prolonged

survival with reduced body weight loss as well as reduced serum levels of alanine aminotransferase, bilirubin, gamma-glutamyl transpeptidase, and alkaline phosphatase, within 4 weeks of transplantation, suggesting that the 3D-bioprinted hepatorganoids alleviated liver injury.

Beyond bioprinting of single-cell-laden bioinks, several studies have used hepatic cell spheroids for the development of 3D liver tissues *in vitro*. The use of cell spheroids protects the cells in the interiors from the mechanical forces during the bioprinting process or the damage caused during the UV-crosslinking process. The cell spheroids can propitiate longer phenotype-retention due to the maintenance of cell signaling, thus being an enabling alternative to single-cell-laden bioinks. A study by Goulart et al. employed extrusion bioprinting of single-cell suspensions and hiPSC-derived hepatocyte cell spheroids encapsulated in hydrogel prepared with alginate (15%) and pluronic F-127 (6%) to obtain donut-shaped liver tissue constructs (Goulart et al., 2019). Both single-cell suspensions and hiPSC-derived hepatocyte cell spheroids composed of hepatoblast cells, hiPSC-derived endothelial cells, and hiPSC-derived mesenchymal cells. Viability and functionality studies after 18 days revealed higher cell viability and hepatic functions such as LDH activity, secretions of urea, albumin, and A1AT in spheroid-liver constructs as compared to those in single-cell-liver constructs. In addition, cells in single-cell-liver constructs exhibited earlier epithelial–mesenchymal transition as compared to that in spheroid-liver constructs, suggesting rapid loss of hepatic phenotypes with single-cell-liver constructs. Similarly, HepG2 spheroids encapsulated in GelMA hydrogel (10%) were directly bioprinted into a microfluidic device to develop a liver-on-a-chip platform (Figure 17.7E) (Bhise et al., 2016). The functionality of the bioprinted hepatic spheroids in the bioreactor was assessed by immunostaining of HepG2 cells for hepatocyte markers such as cytokeratin 18, MRP2 bile canalicular protein, and tight junction protein ZO-1 for up to the 30 days of the culture period (Figure 17.7F) as well as by monitoring the albumin, A1AT, transferrin, and ceruloplasmin secretions (Figure 17.7G). The liver-on-a-chip platform with bioprinted HepG2 spheroids was further applied for testing acute drug hepatotoxicity by treating with acetaminophen at 0–20 mM for 6 days, where the hepatotoxicity of acetaminophen at or above 15 mM of concentrations was revealed.

Vat photopolymerization-based bioprinting is also commonly used to fabricate liver tissue constructs *in vitro*. Grix et al. applied the SLA bioprinting method to produce a hepatic lobule-like structure that composed of 12 channels running from the edges to the central port mimicking the vasculature of the liver (Grix et al., 2018). The channels were bioprinted with degradable polyethyleneglycol-bis-(acryloyloxy acetate) hydrogel (7%) whereas the cellular regions were bioprinted with bioink composed of HepaRG cell- and human hepatic stellate cell-encapsulated GelMA (7%) (Figure 17.8A). These hepatic lobular structures exhibited key liver functions, such as albumin secretion, urea production, and CYP450 enzyme activities (Figure 17.8B). Likewise, Mao et al. successfully used DLP bioprinting to construct liver microtissues using human-induced hepatocyte (hiHep)-encapsulated liver dECM bioink and GelMA (Mao et al., 2020). These liver tissue models were also shown to exhibit liver functions including albumin and urea secretions, as well as CYP450 enzyme activities. In another study by Ma et al., DLP-based bioprinting was employed to develop a 3D triculture hepatic lobule tissue construct with patterned hiPSC-derived hepatic progenitor cells (hiPSC-HPCs) and supporting cells (HUVECs and the ADSCs) in a physiologically relevant architecture using GelMA and glycidyl methacrylate-hyaluronic acid (Ma et al., 2016). This bioprinted microscale hexagonal structure showed morphological organization similar to the native liver microenvironment (Figure 17.8C) and exhibited the expression of liver-specific genes, as well as albumin and urea secretions (Figure 17.8D, E). It was found that among the five CYPs

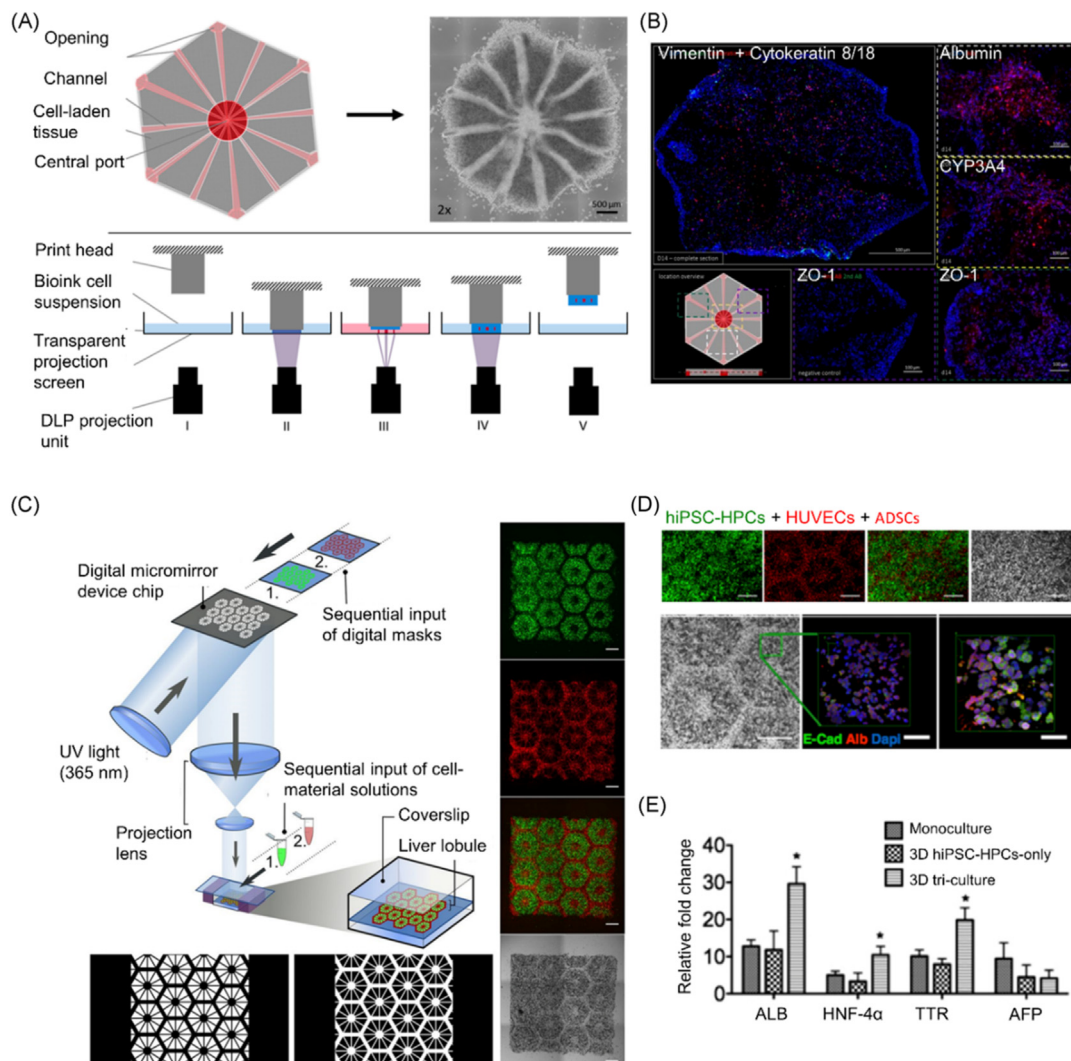


FIGURE 17.8

3D liver constructs fabricated by vat photopolymerization-based bioprinting. (A) Schematics and micrograph of a hepatic lobule-like structure obtained by the SLA bioprinting method. The hepatic lobule-like structure comprising 12 channels that mimic the vasculature of the liver, followed by the schematics showing the DLP bioprinting process. (B) Fluorescence images of bioprinted liver constructs showing the expression of vimentin (green) and cytokeratin 8/18 (red), albumin (red), CYP3A4 (red), and ZO-1 (red) after 14 days of bioprinting. Reproduced under the Creative Commons Attribution License 4.0 for open access articles (Grix et al., 2018). (C) Schematics and real images of liver lobular structures bioprinted by the DLP bioprinting method using iPSC-derived hepatic progenitor cells, HUVECs, and ADSCs embedded in GelMA and glycidyl methacrylate hyaluronic acid. (D) Fluorescent images of bioprinted construct consisting of cell-tracker stained cells on day 10 and immunostained cells for E-cadherin (E-Cad) and albumin (Alb). (E) Gene expression levels of albumin (ALB), hepatocyte nuclear factor-4 α (HNF-4 α), transthyretin (TTR), and α -fetoprotein (AFP) of hiPSC-HPCs in 2D monolayer culture, 3D hiPSC-HPCs-only culture model, and 3D tri-culture system at day 7 after bioprinting. Reproduced under the Creative Commons Attribution License 4.0 for open access articles (Ma et al., 2016).

(CYP1A2, CYP2B6, CYP2C9, CYP2C19, and CYP3A4) that play important roles in liver drug metabolism, the hiPSC-HPCs within the hepatic lobular structure showed a significantly higher expression of CYP3A4, which was estimated to be involved in the metabolism of approximately half the currently used pharmaceutical compounds. Further, it was shown that an antibiotic drug, rifampicin, induced the expressions of CYP3A4, CYP2C9, and CYP2C19 by hiPSC-derived hepatic progenitor cells within the bioprinted hepatic lobular structure.

Recently, the Kenzan bioprinting method has been used to fabricate liver tissue scaffolds for *in vivo* applications. In a study by Yanagi et al., liver bud-like spheroids were generated using human hepatocytes, human mesenchymal stem cells (hMSCs), and HUVECs (Yanagi et al., 2017). These hepatic spheroids were positioned and assembled into a microneedle array. After 2 days, the spheroids were fused together forming the 3D hepatic tissue, which was removed from the microneedle array and maintained in culture for 3 more days. These 3D hepatic tissues were then transplanted into the liver of nude rats. The immunohistochemical analyses after 7 days of transplantation showed the expressions of human CD31 by HUVECs as well as human CYP3A4 and albumin by the human hepatocytes in the graft. Moreover, hematoxylin and eosin (HE) staining and immunohistochemical staining of transplanted graft at day 28 of transplantation revealed the presence of human hepatocytes in the deep area of recipient's liver and human CK19-positive cells in the fibrous area of the transplanted human liver tissue graft, respectively. Similarly, Kizawa et al. employed Kenzan bioprinting to generate a human mini-liver construct by placing HMCS1SA hepatocyte-spheroids on the 9×9 needle array (Kizawa, Nagao, Shimamura, Zhang, & Torii, 2017). The mini-liver tissue constructs formed after the fusion of spheroids were removed from the needle arrays and cultured for more than 2 weeks. These mini-liver constructs exhibited key structural features as shown by HE staining and key functional features of the liver indicated by the expressions of genes related to the metabolism of drugs, glucose, lipid, and urea as well as those encoding transporters and serum proteins.

A step forward, a photolithographic biofabrication method similar to DLP bioprinting was applied to develop 3D human, nonalcoholic, hepatic, steatotic, and fibrotic liver models using HepG2-C3A cell- and HSC (LX-2 cell)-embedded GelMA (5%) (Maharjan, Bonilla, et al., 2021). The 3D hepatic tissue constructs were demonstrated to accumulate fat by HepG2-C3A cells after exposure to saturated palmitic acid (200 μ M) and unsaturated oleic acid (200 μ M). In addition, these scaffolds further exhibited enhanced type I collagen deposition by the HSCs after TGF- β 1 treatment. The free fatty acid-induced steatotic tissues and TGF- β 1-induced fibrotic tissues were further treated with antisteatotic (toyocamycin and obeticholic acid) and antifibrotic drugs (rapamycin and curcumin), respectively. Similarly, spheroid-based extrusion bioprinting was also adopted for modeling the NAFLD (Cartera, Brennerb, Presnell, & Chen, 2021). These examples being a part of the emerging tissue model engineering field are opening up an opportunity for the future generations of liver disease tissues that are amenable to fundamental biological investigations and therapeutics development.

17.7 CHALLENGES AND FUTURE PERSPECTIVES

Although 3D bioprinting provides a rapid fabrication method to develop *in vitro* liver tissue constructs with key structural and functional features of the native liver (Table 17.1), there are still

Table 17.1 Representative 3D-bioprinted liver models.

Method	Bioink	Cells	Structure	Description	Applications	References
Inkjet	Alginate, pluronic	hiPSC-derived hepatic cells	Donut	Bioprinting of single-cell suspension and iPSC-derived hepatocyte cell spheroids for comparative studies	To study liver physiology and in tissue engineering	Goulart et al. (2019)
	Galactosylated alginate	Primary mouse hepatocytes	Rectangular sandwich	Bioprinted hepatocytes sandwiched between two GA-hydrogel sheets for cell adhesion and cell polarity studies	To study the cell adhesion and cell polarity	Arai et al. (2017)
Extrusion	Collagen, pluronic	HepG2	Droplets	Bioprinting of HepG2 cell-suspension with or without pluronic for studying surfactant effects	To study the effects of surfactant on dispensing of living cells through printhead	Parsa et al. (2010)
	Liver dECM, collagen, PCL	HepG2	Grid	Development of liver dECM bioink for liver cell-bioprinting applications	To explore applicability of liver dECM for liver tissue engineering	Lee et al. (2017)
		HepaRG	Grid	Bioprinting of HepaRG cell-laden liver constructs for viral transfection studies	To study adenoviral transduction and viral infection as well as antiviral drug testing	Hiller et al. (2018)
	Alginate, gelatinHuman lung-derived dECM					
	Collagen	hADSCs AHLCs	Grid	Bioprinting of hADSC- and AHLC-laden hepatic blocks for regenerative studies	To study the safety and efficacy of these scaffolds to facilitate the regeneration of damaged liver tissue	Lee et al. (2017)
	GelMA	HepG2	Grid	Bioprinting of HepG2 cell-laden liver tissue constructs for photo-induced crosslinking studies	To study effects of different photoinitiators on cells	Billiet et al. (2014)
	Gelatin	Huh 7	Grids	Bioprinting of Huh 7 cell-laden liver constructs for pore size and contact angle studies	To investigate and optimize the scaffold design for relevant tissue structure and function	Lewis et al. (2018)
	NovoGel	HH, HSCs, and HUVECs	Disc	Bioprinting of HH in the middle, and HSCs and HUVECs in the surroundings for drug-induced liver injury studies	To study drug-induced liver injury	Nguyen et al. (2016) and Norona et al. (2016)
	NovoGel	HH, HSCs, HUVECs, Kupffer cells	Disc	Bioprinting of liver tissues with or without Kupffer cells for studying Kupffer cell function in liver injury	To study fibrosis and evaluate the role of Kupffer cells in liver injury	Norona et al. (2019)
	Alginate, gelatin	HepaRG	Organoid	Bioprinting of hepatorganoids for liver function studies	To investigate liver functions	Yang et al. (2021)

many challenges to be addressed before they become clinically relevant. As the liver is a complex heterogeneous organ with multiple cell types and different levels of microstructures, to completely reproduce the liver microstructure with native liver architecture and functions *in vitro*, an advancement in the current bioprinting method with the improved resolution is required. In addition, the liver consists of abundant vascular and biliary networks that are prerequisites for proper liver functionality. Therefore the inclusion of vascular and biliary systems in liver tissue constructs is critical to recapitulate the dynamic microenvironment of the liver. Nevertheless, *with further progression we anticipate the 3D bioprinting technology to fulfill the goal of successful translation of engineered liver models into clinical applications.*

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QUESTIONS

1. What is 3D bioprinting and how does it work?
2. What are the main types of 3D bioprinting techniques? Explain with their pros and cons?
3. What are the potential benefits of 3D bioprinting?
4. What is bioink? What does one need to keep in mind while selecting biomaterial for 3D bioprinting of liver tissues?
5. What are the different cell types that need to be considered to achieve a fully functional liver tissue?
6. What are the factors that affect cell viability in 3D bioprinting? How does one preserve cell viability as much as possible?
7. What are the applications of 3D-bioprinted liver tissues?
8. What are the main challenges of 3D bioprinting of liver tissues?

References

- Albillos, A., de Gottardi, A., & Rescigno, M. (2020). The gut-liver axis in liver disease: Pathophysiological basis for therapy. *Journal of Hepatology*, 72(3), 558–577.
- Almazroo, O. A., Miah, M. K., & Venkataramanan, R. (2017). Drug metabolism in the liver. *Clinics in Liver Disease*, 21(1), 1–20.
- Arai, K., Yoshida, T., Okabe, M., Goto, M., Mir, T. A., Soko, C., . . . Nakamura, M. (2017). Fabrication of 3D-culture platform with sandwich architecture for preserving liver-specific functions of hepatocytes using 3D bioprinter. *Journal of Biomedical Materials Research. Part A*, 105(6), 1583–1592.
- Asrani, S. K., Devarbhavi, H., Eaton, J., & Kamath, P. S. (2019). Burden of liver diseases in the world. *Journal of Hepatology*, 70(1), 151–171.

- Balmer, M. L., Slack, E., de Gottardi, A., Lawson, M. A., Hapfelmeier, S., Miele, L., . . . Macpherson, A. J. (2014). The liver may act as a firewall mediating mutualism between the host and its gut commensal microbiota. *Science Translational Medicine*, 6(237), 237ra66.
- Ben-Moshe, S., & Itzkovitz, S. (2019). Spatial heterogeneity in the mammalian liver. *Nature Reviews Gastroenterology & Hepatology*, 16(7), 395–410.
- Bhise, N. S., Manoharan, V., Massa, S., Tamayol, A., Ghaderi, M., Miscuglio, M., . . . Khademhosseini, A. (2016). A liver-on-a-chip platform with bioprinted hepatic spheroids. *Biofabrication*, 8(1), 014101.
- Billiet, T., Gevaert, E., De Schryver, T., Cornelissen, M., & Dubruel, P. (2014). The 3D printing of gelatin methacrylamide cell-laden tissue-engineered constructs with high cell viability. *Biomaterials*, 35(1), 49–62.
- Bishop, E. S., Mostafa, S., Pakvasa, M., Luu, H. H., Lee, M. J., Wolf, J. M., . . . Reid, R. R. (2017). 3-D bioprinting technologies in tissue engineering and regenerative medicine: Current and future trends. *Genes & Diseases*, 4(4), 185–195.
- Cao, X., Ashfaq, R., Cheng, F., Maharjan, S., Li, J., Ying, G., . . . Zhang, Y. S. (2019). A tumor-on-a-chip system with bioprinted blood and lymphatic vessel pair. *Advanced Functional Materials*, 29(31), 1807173.
- Cartera, D.E., Brennerb, D., Presnell, S.C., & Chen, A.E. (2021). *Modeling NAFLD using 3D bioprinted human liver tissue*. <<https://organovo.com/modeling-nafl-d-using-3d-bioprinted-human-liver-tissue/>>.
- Casotti, V., & D'Antiga, L. (2019). Basic principles of liver physiology. In L. D'Antiga (Ed.), *Pediatric hepatology and liver transplantation* (pp. 21–39). Springer.
- Chang, C. C., Boland, E. D., Williams, S. K., & Hoying, J. B. (2011). Direct-write bioprinting three-dimensional biohybrid systems for future regenerative therapies. *Journal of Biomedical Materials Research. Part B, Applied Biomaterials*, 98(1), 160–170.
- Chang, R., Nam, J., & Sun, W. (2008). Effects of dispensing pressure and nozzle diameter on cell survival from solid freeform fabrication-based direct cell writing. *Tissue Engineering. Part A*, 14(1), 41–48.
- Di Ciaula, A., Baj, J., Garruti, G., Celano, G., De Angelis, M., Wang, H. H., . . . Portincasa, P. (2020). Liver steatosis, gut-liver axis, microbiome and environmental factors. A never-ending bidirectional cross-talk. *Journal of Clinical Medicine*, 9(8), 2648.
- Cui, X., Boland, T., D'Lima, D. D., & Lotz, M. K. (2012). Thermal inkjet printing in tissue engineering and regenerative medicine. *Recent Patents on Drug Delivery & Formulation*, 6(2), 149–155.
- Cui, H., Nowicki, M., Fisher, J. P., & Zhang, L. G. (2017). 3D bioprinting for organ regeneration. *Advanced Healthcare Materials*, 6(1). Available from <https://doi.org/10.1002/adhm.201601118>.
- Daly, A. C., Davidson, M. D., & Burdick, J. A. (2021). 3D bioprinting of high cell-density heterogeneous tissue models through spheroid fusion within self-healing hydrogels. *Nature Communications*, 12(1), 753.
- Diamantides, N., Dugopolski, C., Blahut, E., Kennedy, S., & Bonassar, L. J. (2019). High density cell seeding affects the rheology and printability of collagen bioinks. *Biofabrication*, 11(4), 045016.
- El-Serag, H. B. (2012). Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology*, 142(6), 1264–1273.e1.
- Elpek, G. (2014). Cellular and molecular mechanisms in the pathogenesis of liver fibrosis: An update. *World Journal of Gastroenterology: WJG*, 20(23), 7260–7276.
- Faulkner-Jones, A., Fyfe, C., Cornelissen, D. J., Gardner, J., King, J., Courtney, A., & Shu, W. (2015). Bioprinting of human pluripotent stem cells and their directed differentiation into hepatocyte-like cells for the generation of mini-livers in 3D. *Biofabrication*, 7(4), 044102.
- Fausto, N., & Campbell, J. S. (2003). The role of hepatocytes and oval cells in liver regeneration and repopulation. *Mechanisms of Development*, 120(1), 117–130.
- Friedman, S. L. (2008). Hepatic stellate cells: Protean, multifunctional, and enigmatic cells of the liver. *Physiological Reviews*, 88(1), 125–172.
- Gabriel, S., Maroney, T. P., & Ringe, B. H. (2007). Hepatic artery-portal vein fistula formation after percutaneous liver biopsy in a living liver donor. *Transplantation Proceedings*, 39(5), 1707–1709.

- Gao, G., Yonezawa, T., Hubbell, K., Dai, G., & Cui, X. (2015). Inkjet-bioprinted acrylated peptides and PEG hydrogel with human mesenchymal stem cells promote robust bone and cartilage formation with minimal printhead clogging. *Biotechnology Journal*, 10(10), 1568–1577.
- Gilgenkrantz, H., & Collin de l'Hortet, A. (2018). Understanding liver regeneration: From mechanisms to regenerative medicine. *The American Journal of Pathology*, 188(6), 1316–1327.
- Gilgenkrantz, H., & Collin de l'Hortet, A. (2011). New insights into liver regeneration. *Clinics and Research in Hepatology and Gastroenterology*, 35(10), 623–629.
- Gissen, P., & Arias, I. M. (2015). Structural and functional hepatocyte polarity and liver disease. *Journal of Hepatology*, 63(4), 1023–1037.
- Gluchowski, N. L., Becuwe, M., Walther, T. C., & Farese, R. V., Jr. (2017). Lipid droplets and liver disease: From basic biology to clinical implications. *Nature Reviews. Gastroenterology & Hepatology*, 14(6), 343–355.
- Goulart, E., de Caires-Junior, L. C., Telles-Silva, K. A., Araujo, B. H. S., Rocco, S. A., Sforca, M., ... Zatz, M. (2019). 3D bioprinting of liver spheroids derived from human induced pluripotent stem cells sustain liver function and viability in vitro. *Biofabrication*, 12(1), 015010.
- Gracia-Sancho, J., Marrone, G., & Fernández-Iglesias, A. (2019). Hepatic microcirculation and mechanisms of portal hypertension. *Nature Reviews Gastroenterology & Hepatology*, 16(4), 221–234.
- Grix, T., Ruppelt, A., Thomas, A., Amler, A.-K., Noichl, B. P., Lauster, R., & Kloke, L. (2018). Bioprinting perfusion-enabled liver equivalents for advanced organ-on-a-chip applications. *Genes*, 9(4), 176.
- Gudapati, H., Dey, M., & Ozbolat, I. (2016). A comprehensive review on droplet-based bioprinting: Past, present and future. *Biomaterials*, 102, 20–42.
- Hajdarevic, B., Vehabovic, I., Catic, T., & Masic, I. (2020). The role of diet therapy in the treatment of liver disease. *Materia Sociomedica*, 32(3), 200–206.
- Heinrich, M. A., Liu, W., Jimenez, A., Yang, J., Akpek, A., Liu, X., ... Zhang, Y. S. (2019). 3D bioprinting: From benches to translational applications. *Small (Weinheim an der Bergstrasse, Germany)*, 15(23), e1805510.
- Heydari, Z., Najimi, M., Mirzaei, H., Shpichka, A., Ruoss, M., Farzaneh, Z., ... Vosough, M. (2020). Tissue engineering in liver regenerative medicine: Insights into novel translational technologies. *Cells*, 9(2), 304.
- Hiller, T., Berg, J., Elomaa, L., Röhrs, V., Ullah, I., Schaar, K., ... Kurreck, J. (2018). Generation of a 3D liver model comprising human extracellular matrix in an alginate/gelatin-based bioink by extrusion bioprinting for infection and transduction studies. *International Journal of Molecular Sciences*, 19(10), 3129.
- Ingber, D. E., Mow, V. C., Butler, D., Niklason, L., Huard, J., Mao, J., ... Vunjak-Novakovic, G. (2006). Tissue engineering and developmental biology: Going biomimetic. *Tissue Engineering*, 12(12), 3265–3283.
- Jadlowiec, C. C., & Taner, T. (2016). Liver transplantation: Current status and challenges. *World Journal of Gastroenterology*, 22(18), 4438.
- Jeong, H.-J., Nam, H., Jang, J., & Lee, S.-J. (2020). 3D bioprinting strategies for the regeneration of functional tubular tissues and organs. *Bioengineering (Basel)*, 7(2), 32.
- Jeon, H., Kang, K., Park, S. A., Kim, W. D., Paik, S. S., Lee, S. H., ... Choi, D. (2017). Generation of multi-layered 3D structures of HepG2 cells using a bio-printing technique. *Gut and Liver*, 11(1), 121–128.
- Jeon, O., Lee, Y. B., Jeong, H., Lee, S. J., Wells, D., & Alsberg, E. (2019). Individual cell-only bioink and photocurable supporting medium for 3D printing and generation of engineered tissues with complex geometries. *Materials Horizons*, 6(8), 1625–1631.
- Jungermann, K., & Katz, N. (1989). Functional specialization of different hepatocyte populations. *Physiological Reviews*, 69(3), 708–764.
- Kang, Y., & Elia, E. (2016). Anesthesia management of liver transplantation. *Contemporary Liver Transplantation*, 143–187.

- Kang, D., Hong, G., An, S., Jang, I., Yun, W.-S., Shim, J.-H., & Jin, S. (2020). Bioprinting of multiscaled hepatic lobules within a highly vascularized construct. *Small*, 16(13), 1905505.
- Kietzmann, T. (2017). Metabolic zonation of the liver: The oxygen gradient revisited. *Redox Biology*, 11, 622–630.
- Kim, J. D., Choi, J. S., Kim, B. S., Chan Choi, Y., & Cho, Y. W. (2010). Piezoelectric inkjet printing of polymers: Stem cell patterning on polymer substrates. *Polymer*, 51(10), 2147–2154.
- Kim, Y., Kang, K., Jeong, J., Paik, S. S., Kim, J. S., Park, S. A., ... Choi, D. (2017). Three-dimensional (3D) printing of mouse primary hepatocytes to generate 3D hepatic structure. *Annals of Surgical Treatment and Research*, 92(2), 67–72.
- Kim, Y., Kang, K., Yoon, S., Kim, J. S., Park, S. A., Kim, W. D., ... Choi, D. (2018). Prolongation of liver-specific function for primary hepatocytes maintenance in 3D printed architectures. *Organogenesis*, 14(1), 1–12.
- Kizawa, H., Nagao, E., Shimamura, M., Zhang, G., & Torii, H. (2017). Scaffold-free 3D bio-printed human liver tissue stably maintains metabolic functions useful for drug discovery. *Biochemistry and Biophysics Reports*, 10, 186–191.
- Kozeniecki, M., Ludke, R., Kerner, J., & Patterson, B. (2020). Micronutrients in liver disease: Roles, risk factors for deficiency, and recommendations for supplementation. *Nutrition in Clinical Practice*, 35(1), 50–62.
- Kryou, C., Leva, V., Chatzipetrou, M., & Zergioti, I. (2019). Bioprinting for liver transplantation. *Bioengineering (Basel, Switzerland)*, 6(4), 95.
- Kumar, H., & Kim, K. (2020). Stereolithography 3D bioprinting. *Methods in Molecular Biology*, 2140, 93–108.
- Lee, H., Han, W., Kim, H., Ha, D. H., Jang, J., Kim, B. S., & Cho, D. W. (2017). Development of liver decellularized extracellular matrix bioink for three-dimensional cell printing-based liver tissue engineering. *Biomacromolecules*, 18(4), 1229–1237.
- Lee, J.-S., Yoon, H., Yoon, D., Kim, G. H., Yang, H. T., & Chun, W. (2017). Development of hepatic blocks using human adipose tissue-derived stem cells through three-dimensional cell printing techniques. *Journal of Materials Chemistry B*, 5(5), 1098–1107.
- Lewis, P. L., Green, R. M., & Shah, R. N. (2018). 3D-printed gelatin scaffolds of differing pore geometry modulate hepatocyte function and gene expression. *Acta Biomaterialia*, 69, 63–70.
- Liu, W., Heinrich, M. A., Zhou, Y., Akpek, A., Hu, N., Liu, X., ... Zhang, Y. S. (2017). Extrusion bioprinting of shear-thinning gelatin methacryloyl bioinks. *Advanced Healthcare Materials*, 6(12). Available from <https://doi.org/10.1002/adhm.201601451>.
- Li, W., Mille, L. S., Robledo, J. A., Uribe, T., Huerta, V., & Zhang, Y. S. (2020). Recent advances in formulating and processing biomaterial inks for vat polymerization-based 3D printing. *Advanced Healthcare Materials*, 9(15), 2000156.
- MacParland, S. A., Liu, J. C., Ma, X.-Z., Innes, B. T., Bartczak, A. M., Gage, B. K., ... McGilvray, I. D. (2018). Single cell RNA sequencing of human liver reveals distinct intrahepatic macrophage populations. *Nature Communications*, 9(1), 4383.
- Maharjan, S., Alva, J., Cámara, C., Rubio, A. G., Hernández, D., Delavaux, C., ... Zhang, Y. S. (2021). Symbiotic photosynthetic oxygenation within 3D-bioprinted vascularized tissues. *Matter*, 4(1), 217–240.
- Maharjan, S., Bonilla, D., Sindurakar, P., Li, H., Li, W., Duarte, S., ... Zhang, Y. S. (2021). 3D human nonalcoholic hepatic steatosis and fibrosis models. *Bio-Design and Manufacturing*, 4, 157–170.
- Mansour, D., & McPherson, S. (2018). Management of decompensated cirrhosis. *Clinical Medicine (London)*, 18(Suppl 2), s60–s65.
- Mao, Q., Wang, Y., Li, Y., Juengpanich, S., Li, W., Chen, M., ... Cai, X. (2020). Fabrication of liver microtissue with liver decellularized extracellular matrix (dECM) bioink by digital light processing (DLP) bioprinting. *Materials Science and Engineering C: Materials for Biological Applications*, 109, 110625.

- Marchisello, S., Di Pino, A., Scicali, R., Urbano, F., Piro, S., Purrello, F., & Rabuazzo, A. M. (2019). Pathophysiological, molecular and therapeutic issues of nonalcoholic fatty liver disease: An overview. *International Journal of Molecular Sciences*, 20(8), 1948.
- Matsusaki, M., Sakaue, K., Kadowaki, K., & Akashi, M. (2013). Three-dimensional human tissue chips fabricated by rapid and automatic inkjet cell printing. *Advanced Healthcare Materials*, 2(4), 534–539.
- Matyas, C., Haskó, G., Liaudet, L., Trojnar, E., & Pacher, P. (2021). Interplay of cardiovascular mediators, oxidative stress and inflammation in liver disease and its complications. *Nature Reviews Cardiology*, 18(2), 117–135.
- Mazzocchi, A., Devarasetty, M., Huntwork, R., Soker, S., & Skardal, A. (2018). Optimization of collagen type I-hyaluronan hybrid bioink for 3D bioprinted liver microenvironments. *Biofabrication*, 11(1), 015003.
- Ma, X., Qu, X., Zhu, W., Li, Y. S., Yuan, S., Zhang, H., . . . Chen, S. (2016). Deterministically patterned biomimetic human iPSC-derived hepatic model via rapid 3D bioprinting. *Proceedings of the National Academy of Sciences of the United States of America*, 113(8), 2206–2211.
- Ma, L., Wu, Y., Li, Y., Aazmi, A., Zhou, H., Zhang, B., & Yang, H. (2020). Current advances on 3D-bioprinted liver tissue models. *Advanced Healthcare Materials*, 9(24), e2001517.
- Ma, X., Yu, C., Wang, P., Xu, W., Wan, X., Lai, C. S. E., . . . Chen, S. (2018). Rapid 3D bioprinting of decellularized extracellular matrix with regionally varied mechanical properties and biomimetic microarchitecture. *Biomaterials*, 185, 310–321.
- Michalopoulos, G. K. (2017). Hepatostat: Liver regeneration and normal liver tissue maintenance. *Hepatology*, 65(4), 1384–1392.
- Michalopoulos, G. K., & Bhushan, B. (2021). Liver regeneration: Biological and pathological mechanisms and implications. *Nature Reviews Gastroenterology & Hepatology*, 18(1), 40–55.
- Mironov, V., Visconti, R. P., Kasyanov, V., Forgacs, G., Drake, C. J., & Markwald, R. R. (2009). Organ printing: Tissue spheroids as building blocks. *Biomaterials*, 30(12), 2164–2174.
- Moroni, L., Burdick, J. A., Highley, C., Lee, S. J., Morimoto, Y., Takeuchi, S., & Yoo, J. J. (2018). Biofabrication strategies for 3D in vitro models and regenerative medicine. *Nature Reviews Materials*, 3(5), 21–37.
- Murata, D., Arai, K., & Nakayama, K. (2020). Scaffold-free bio-3D printing using spheroids as “bio-inks” for tissue (re-)construction and drug response tests. *Advanced Healthcare Materials*, 9(15), e1901831.
- Murphy, S. V., & Atala, A. (2014). 3D bioprinting of tissues and organs. *Nature Biotechnology*, 32(8), 773–785.
- Neiman, J. A., Raman, R., Chan, V., Rhoads, M. G., Raredon, M. S., Velazquez, J. J., . . . Griffith, L. G. (2015). Photopatterning of hydrogel scaffolds coupled to filter materials using stereolithography for perfused 3D culture of hepatocytes. *Biotechnology and Bioengineering*, 112(4), 777–787.
- Nguyen-Lefebvre, A. T., & Horuzsko, A. (2015). Kupffer cell metabolism and function. *Journal of Enzymology and Metabolism*, 1(1), 101.
- Nguyen, D. G., Funk, J., Robbins, J. B., Crogan-Grundy, C., Presnell, S. C., Singer, T., & Roth, A. B. (2016). Bioprinted 3D primary liver tissues allow assessment of organ-level response to clinical drug induced toxicity in vitro. *PLoS One*, 11(7), e0158674.
- Norona, L. M., Nguyen, D. G., Gerber, D. A., Presnell, S. C., & LeCluyse, E. L. (Eds.), (2016). Editor's highlight: Modeling compound-induced fibrogenesis in vitro using three-dimensional bioprinted human liver tissues. *Toxicological Sciences: An Official Journal of the Society of Toxicology*, 154(2), 354–367.
- Norona, L. M., Nguyen, D. G., Gerber, D. A., Presnell, S. C., Mosedale, M., & Watkins, P. B. (2019). Bioprinted liver provides early insight into the role of Kupffer cells in TGF- β 1 and methotrexate-induced fibrogenesis. *PLoS One*, 14(1), e0208958.
- Norotte, C., Marga, F. S., Niklason, L. E., & Forgacs, G. (2009). Scaffold-free vascular tissue engineering using bioprinting. *Biomaterials*, 30(30), 5910–5917.

- Osidak, E. O., Kozhukhov, V. I., Osidak, M. S., & Domogatsky, S. P. (2020). Collagen as bioink for bioprinting: A comprehensive review. *International Journal of Bioprinting*, 6(3), 270.
- Ozbolat, I. T., & Hospodiuk, M. (2016). Current advances and future perspectives in extrusion-based bioprinting. *Biomaterials*, 76, 321–343.
- Ozbolat, I. T., Peng, W., & Ozbolat, V. (2016). Application areas of 3D bioprinting. *Drug Discovery Today*, 21(8), 1257–1271.
- Parsa, S., Gupta, M., Loizeau, F., & Cheung, K. C. J. B. (2010). Effects of surfactant and gentle agitation on inkjet dispensing of living cells. *Biofabrication*, 2(2), 025003.
- Pi, Q., Maharjan, S., Yan, X., Liu, X., Singh, B., van Genderen, A. M., . . . Zhang, Y. S. (2018). Digitally tunable microfluidic bioprinting of multilayered cannular tissues. *Advanced Materials*, 30(43), e1706913.
- Poisson, J., Lemoine, S., Boulanger, C., Durand, F., Moreau, R., Valla, D., & Rautou, P. E. (2017). Liver sinusoidal endothelial cells: Physiology and role in liver diseases. *Journal of Hepatology*, 66(1), 212–227.
- Poomathi, N., Singh, S., Prakash, C., Subramanian, A., Sahay, R., Cinappan, A., & Ramakrishna, S. J. R. P. J. (2020). 3D printing in tissue engineering: A state of the art review of technologies and biomaterials. *Rapid Prototyping Journal*. Available from <https://doi.org/10.1108/RPJ-08-2018-021>.
- Reed, E. J., Klumb, L., Koobatian, M., & Viney, C. (2009). Biomimicry as a route to new materials: What kinds of lessons are useful? *Philosophical Transactions. Series A, Mathematical, Physical, and Engineering Sciences*, 367(1893), 1571–1585.
- Saunders, R. E., Gough, J. E., & Derby, B. (2008). Delivery of human fibroblast cells by piezoelectric drop-on-demand inkjet printing. *Biomaterials*, 29(2), 193–203.
- Saxena, R., Theise, N. D., & Crawford, J. M. (1999). Microanatomy of the human liver—Exploring the hidden interfaces. *Hepatology*, 30(6), 1339–1346.
- Schulze, R. J., Schott, M. B., Casey, C. A., Tuma, P. L., & McNiven, M. A. (2019). The cell biology of the hepatocyte: A membrane trafficking machine. *Journal of Cell Biology*, 218(7), 2096–2112.
- Schuppan, D., & Afdhal, N. H. (2008). Liver cirrhosis. *Lancet (London, England)*, 371(9615), 838–851.
- Sepanlou, S. G., Safiri, S., Bisignano, C., Ikuta, K. S., Merat, S., Saberifiroozi, M., . . . Colombara, D. V. (2020). The global, regional, and national burden of cirrhosis by cause in 195 countries and territories, 1990–2017: A systematic analysis for the Global Burden of Disease Study 2017. *The Lancet. Gastroenterology & Hepatology*, 5(3), 245–266.
- Shah, N. L., & Caldwell, S. H. (2016). Assessing the risk of bleeding and clotting in cirrhosis. *Clinical Liver Disease*, 7(2), 26–28.
- Shim, J. H., Kim, J. Y., Park, M., Park, J., & Cho, D. W. (2011). Development of a hybrid scaffold with synthetic biomaterials and hydrogel using solid freeform fabrication technology. *Biofabrication*, 3(3), 034102.
- Stec, D. E., John, K., Trabbic, C. J., Luniwal, A., Hankins, M. W., Baum, J., & Hinds, T. D., Jr. (2016). Bilirubin binding to PPAR α inhibits lipid accumulation. *PLoS One*, 11(4), e0153427.
- Tanaka, M., & Miyajima, A. (2016). Liver regeneration and fibrosis after inflammation. *Inflammation and Regeneration*, 36(1), 19.
- Trefts, E., Gannon, M., & Wasserman, D. H. (2017). The liver. *Current Biology*, 27(21), R1147–R1151.
- Wang, X., Yan, Y., Pan, Y., Xiong, Z., Liu, H., Cheng, J., . . . Lu, Q. (2006). Generation of three-dimensional hepatocyte/gelatin structures with rapid prototyping system. *Tissue Engineering*, 12(1), 83–90.
- Weiskirchen, R., & Tacke, F. (2014). Cellular and molecular functions of hepatic stellate cells in inflammatory responses and liver immunology. *Hepatobiliary Surgery and Nutrition*, 3(6), 344–363.
- Wijshoff, H. (2010). The dynamics of the piezo inkjet printhead operation. *Physics Reports*, 491(4), 77–177.
- Wu, Y., Lin, Z. Y., Wenger, A. C., Tam, K. C., & Tang, X. (2018). 3D bioprinting of liver-mimetic construct with alginate/cellulose nanocrystal hybrid bioink. *Bioprinting*, 9, 1–6.

- Wu, Y., Wenger, A., Golzar, H., & Tang, X. (2020). 3D bioprinting of bicellular liver lobule-mimetic structures via microextrusion of cellulose nanocrystal-incorporated shear-thinning bioink. *Scientific Reports*, 10(1), 20648.
- Xie, Z., Gao, M., Lobo, A. O., & Webster, T. J. (2020). 3D bioprinting in tissue engineering for medical applications: The classic and the hybrid. *Polymers (Basel)*, 12(8), 1717.
- Xu, T., Zhao, W., Zhu, J. M., Albanna, M. Z., Yoo, J. J., & Atala, A. (2013). Complex heterogeneous tissue constructs containing multiple cell types prepared by inkjet printing technology. *Biomaterials*, 34(1), 130–139.
- Yanagi, Y., Nakayama, K., Taguchi, T., Enosawa, S., Tamura, T., Yoshimaru, K., . . . Kobayashi, E. (2017). In vivo and ex vivo methods of growing a liver bud through tissue connection. *Scientific Reports*, 7(1), 14085.
- Yang, H., Sun, L., Pang, Y., Hu, D., Xu, H., Mao, S., . . . Mao, Y. (2021). Three-dimensional bioprinted hepatorganoids prolong survival of mice with liver failure. *Gut*, 70(3), 567–574.
- Ying, G.-L., Jiang, N., Maharjan, S., Yin, Y.-X., Chai, R.-R., Cao, X., . . . Zhang, Y. S. (2018). Aqueous two-phase emulsion bioink-enabled 3D bioprinting of porous hydrogels. *Advanced Materials*, 30(50), e1805460.
- Ying, G., Jiang, N., Yu, C., & Zhang, Y. S. (2018). Three-dimensional bioprinting of gelatin methacryloyl (GelMA). *Bio-Design and Manufacturing*, 1(4), 215–224.
- Ying, G., Manríquez, J., Wu, D., Zhang, J., Jiang, N., Maharjan, S., . . . Zhang, Y. S. (2020). An open-source handheld extruder loaded with pore-forming bioink for in situ wound dressing. *Materials Today Bio*, 8, 100074.
- Yu, Y., Moncal, K. K., Li, J., Peng, W., Rivero, I., Martin, J. A., & Ozbolat, I. T. (2016). Three-dimensional bioprinting using self-assembling scalable scaffold-free “tissue strands” as a new bioink. *Scientific Reports*, 6(1), 28714.
- Zeilinger, K., Freyer, N., Damm, G., Seehofer, D., & Knöspel, F. (2016). Cell sources for in vitro human liver cell culture models. *Experimental Biology and Medicine (Maywood, NJ)*, 241(15), 1684–1698.
- Zhang, J., Hu, Q., Wang, S., Tao, J., & Gou, M. (2019). Digital light processing based three-dimensional printing for medical applications. *International Journal of Bioprinting*, 6(1), 242.
- Zhang, Y. S., Yue, K., Aleman, J., Mollazadeh-Moghaddam, K., Bakht, S. M., Yang, J., . . . Khademhosseini, A. (2017). 3D bioprinting for tissue and organ fabrication. *Annals of Biomedical Engineering*, 45(1), 148–163.
- Zhou, W.-C., Zhang, Q.-B., & Qiao, L. (2014). Pathogenesis of liver cirrhosis. *World Journal of Gastroenterology: WJG*, 20(23), 7312–7324.

ORGAN PRINTING

18

Robert C. Chang and Filippos Tourlomousis*Department of Mechanical Engineering, Stevens Institute of Technology, Hoboken, NJ, United States*

18.1 INTRODUCTION

Our enhanced understanding of the fundamental biological sciences, primarily the interplay between biological cells and integrative biological, chemical, and structural cues within the *in vivo* milieu or natural three-dimensional (3D) microenvironment, demands a defined role for engineering design and manufacturing to meet the challenges presented by increasingly complex biological problems. Organ printing in tissue engineering is the application of additive, computer-aided manufacturing process technologies toward the layered, patterned deposition of complex 3D cell-bearing biological structures with biomolecular and biopolymer material integration. Organ printing therefore encapsulates an ever-expanding range of enabling bioadditive manufacturing processes and strategies that show great promise in regenerative medicine applications where novel fabrication, in conjunction with rapidly evolving stem cell technologies, is envisioned to address the challenge of limited donor grafts for functional organ repair and replacement.

The term organ printing has previously been more narrowly defined as “a biomedical variant of rapid prototyping technology or computer-aided robotic layer-by-layer additive biofabrication of 3D human tissues and organs using self-assembling tissue spheroids as building blocks (Mironov et al., 2003; Mironov et al., 2008; Mironov et al., 2009; Mironov et al., 2011).” Such a definition is rooted in developmental biology principles in which the self-assembly process refers to “one in which humans are not actively involved, in which atoms, molecules, aggregates of molecules, and components arrange themselves into ordered functioning entities without human intervention (Whitesides and Grzybowski, 2002).” Accordingly, the raw material or tissue spheroid “bioink” for bioprinting is initially preprocessed by achieving high-density cell aggregates via traditional hanging drop methods, high-throughput digital microfluidics, and other scalable fabrication methods of tissue spheroids with self-organizing properties (Rezende et al., 2013). More broadly defined in the context of tissue engineering, the autonomous organization of biological entities along the continuum of biological scales can be described conceptually as man kick-starting nature’s mechanism of constructing an innately coordinated system whereby “people may design the process, and they may launch it, but once underway, it proceeds according to its own internal plan, either toward an energetically stable form or toward some system whose form and function are encoded in its individual parts (Whitesides, 1995).” Therefore, given the significant insight we’ve gained in systematically engineering cell-instructive microenvironments, we

will adopt this latter definition of self-assembly as an organizing principle for enabling bioadditive manufacturing technologies driven by either (1) scaffold-free approaches, (2) scaffold-directed approaches, or (3) a hybrid of these prevailing approaches (Burdick and Vunjak-Novakovic, 2009). This inclusive definition of organ printing captures layer-by-layer fabrication technologies capable of satisfying the criteria of directly incorporating or seeding engineered constructs with high-density, concentrated cell suspensions toward *de novo* complex tissue form and function in organs.

18.1.1 ORGAN PRINTING TECHNIQUES

Current layer-by-layer technologies developed and implemented for the purpose of digitally printing human organs can be divided into three broad categories: (1) microextrusion-based printing, (2) ink-jet-based printing, and (3) laser-based printing. Other printing techniques have also emerged and their attributes will be briefly described in this chapter. In each of these categories, the term printing refers specifically to the direct patterning or deposition of cells either with or without a carrying matrix biopolymer. As part of the broadening bioadditive manufacturing armamentarium, these rapid prototyping (RP) or solid freeform fabrication (SFF) techniques are capable of varying degrees of resolution, reproducibly patterned architectures within a bulk construct, as well as scalable manufacturing of clinically relevant defect sizes.

18.1.1.1 Microextrusion-based Printing

A promising freeform fabrication process, which has perhaps gained the widest adoption for organ printing applications, is microextrusion-based printing, sometimes referred to in the literature as bioplotting or biodispensing. Microextrusion-based printing represents a progression from fused deposition modeling (FDM, our traditional understanding of 3D printing) that expands the catalog of candidate processed materials into fibers or filaments beyond synthetic polymer melts to include hydrogels and incorporate cells into the printing step. Furthermore, while FDM toward fabricating polycaprolactone (PCL) thermoplastic and composite material 3D scaffolds has found wide application in hard tissue applications, microextrusion-based printing is capable of building layered structures at cell-friendly temperatures amenable for processing biopolymer materials laden with cells and biological factors toward solid organ printing. Target mammalian cell types for microextrusion-based printing include liver, bone, heart, vascular lining, and cartilage (Chang et al., 2008a; Khalil and Sun, 2009; Cohen et al., 2009; Gaetani et al., 2012; Shim et al., 2011; Smith et al., 2004; Wang et al., 2006; Yan et al., 2005). A wide number of both natural and synthetic polymer hydrogel materials have been used in microextrusion-based printing including alginate, agarose, Matrigel®, fibrinogen, gelatin, collagen, chitosan, and polyethylene glycol (PEG) (Chang et al., 2008a; Khalil and Sun, 2009; Cohen et al., 2009; Gaetani et al., 2012; Shim et al., 2011; Smith et al., 2004; Wang et al., 2006; Yan et al., 2005; Fedorovich et al., 2008; Fedorovich et al., 2011a; Ahn and Koh, 2010; Smith et al., 2007; Maher et al., 2009). In microextrusion-based printing methods, cells and hydrogel precursors are dispensed from a syringe reservoir or micronozzle containing cell suspension via a syringe pump piston or pneumatic microvalve, respectively. A significant advantage of this method is the facile incorporation of cells and biological signaling molecules that can be spatially patterned within a 3D layered construct. Furthermore, foundational work has been done in experimentally quantifying and predictively modeling the postprint viability

and functional retention of various cell types following process-induced mechanical loading. For example, the authors have examined the effect of dispensing pressure and nozzle tip diameter on the postprint viability of HepG2 liver cells encapsulated within alginate, demonstrating a quantifiable loss of cell viability due to process-induced mechanical damage with some cells showing recovery over a short-duration study period (Chang et al., 2008a). Others have shown with cross-validating cell damage models of printed Schwann cells and 3T3 fibroblasts that differential postprint viability is observed in tapered versus cylindrical needle tip geometries (Li et al., 2011). While microextrusion-based postprinted aggregate cell functional retention has been demonstrated in various cell types for narrow process windows, more systematic study to quantify and isolate these process-induced effects in the context of diverse material parameters (e.g. viscosity) would contribute to fundamental understanding of these mechanical processes toward widening the permissible process windows with predictable biological postprint outcomes. This understanding of process-induced mechanical forces in microextrusion-based printing will become even more critical as (1) the requirement for high-density cell aggregates and tissue spheroids becomes paramount in organ printing applications, and (2) stem cells sensitive to mechanical cues become a mainstay in microextrusion-based organ printing for regenerative medicine. The latter challenge of predicting the effect of process-induced mechanical cues on stem cell differentiation also represents an opportunity to reliably guide cell fates for the first time with bioadditive manufacturing systems. Compared with the other organ techniques discussed in this chapter, microextrusion-based printing offers the lowest resolution on the order of 100–200 μm .

18.1.1.2 Ink-jet-based Printing

Drop-on-demand or ink-jet-based printing has been adapted from commercial desktop printers to precisely deposit and pattern biological cells and materials with picoliter-sized volumes (Sekula et al., 2008). Relative to the microextrusion-based printing methods, ink-jet-based printers are able to achieve feature sizes on the order of 20–100 μm (Melchels et al., 2012). This approach entails repurposing desktop printer ink cartridges by replacing the ink in the material reservoir with bioink (i.e. aqueous cell solutions) to print microscale cell droplets (Xu et al., 2005; Roth et al., 2004). Since ink-jet printing was initially aimed at fabricating two-dimensional systems (i.e. a single layer of droplets for surface patterning applications), the feature sizes have been determined to be primarily dictated by the size of the printed drop in flight and its contact angle on the surface upon impact (Stringer and Derby, 2010). The resultant resolution is that the smallest drops attainable through an ink-jet-based process are ~ 1 pl volumes with an equivalent radius of less than 10 μm . Therefore, on prescribed hydrophilic surfaces, minimum features sizes on the order of single-cell dimensions are achievable with this technique. In order to create a layered configuration of cells with encapsulating biopolymers, others have designed an elevator chamber model for ink-jet-based printer in which robotic control of a metal elevator rod is regulated using a step motor for precise positioning (Boland et al., 2006). In this study, precise volumes of chemical cross-linking solution are ejected from the ink-jet head or nozzle onto the motorized elevator submerged within a solution of uncrosslinked hydrogel precursor intermixed with cells homogeneously distributed. While this approach successfully circumvents the undesired effect of cells being subjected to process-induced mechanical loading (e.g. shear stresses and impact forces) through an ink-jet cartridge orifice, the ability to spatially pattern, using multiple ink-jet cartridges as well as multicellular and multimaterial constructs with functionally gradient distributions, is limited by the initial cell and prepolymer material configuration residing

in the elevator chamber. Alternatively, others have implemented electrostatically driven ink-jet-based printer systems to deposit cell suspensions with single-cell seeding control (Nakamura *et al.*, 2010). Others have simultaneously ejected hydrogels and cells from an ink-jet nozzle into chemical cross-linking solutions (Nishiyama *et al.*, 2009). While these high-resolution techniques enable cells to be spatially patterned with heterogeneous distribution by modulating the ejection frequency and multiple material heads, the question of cell viability from the jetting of high-density cell suspensions through a small cartridge orifice remains unanswered. Furthermore, on one hand, ink-jet printing cell suspensions in media at high ejection frequencies and smaller drop feature sizes are hampered by evaporative water losses and a lack of a material support apparatus. On the other hand, ink-jet printing cells with polymer materials place restrictions on the types of polymers that can be processed to low-viscosity, low-strength hydrogel materials with a viscosity ceiling of approximately 30 mPa*s (Melchels *et al.*, 2012). A corollary of these material handling restrictions is the challenge with inkjet-based printing to achieve buildup in 3D for organ printing.

18.1.1.3 Laser-based Printing

Laser-based printing, sometimes referred to in the literature as laser-assisted bioprinting or laser forward transfer, is a nozzle-free printing technique that enables high-resolution patterning of cells (Guillemot *et al.*, 2010; Gruene *et al.*, 2011; Koch *et al.*, 2010; Mezel *et al.*, 2010). In laser-based printing system configurations, a collector plate with tunable wetting properties is first coated with encapsulating polymer droplets loaded with cells. Either UV or IR laser is then focused with a microscope objective lens onto an absorbing thin layer film typically composed of Au, Ag, or Ti that shields the cell-polymer droplets from direct laser interactions. Upon reaching a minimum threshold energy of the nanosecond pulsed laser, microscale droplet deposition is achieved. Key process parameters in laser-based printing that dictate droplet feature sizes include the thickness of the absorbing layer, distance between the absorbing layer and collector plate, wavelength or energy of the laser pulses, and the optical properties of the polymer material. The significant advantages of laser-based printing include the small feature sizes attainable and the ability to pattern cells without subjecting them to the mechanical forces inherent in nozzle-based, ink-jet-based, and microextrusion-based printing methods. A nozzle-free configuration also circumvents occlusion issues that beset ink-jet-based and microextrusion-based techniques. Akin to ink-jet-based printing, however, scale-up of laser-based printed constructs is challenging due to physical constraints in the Z-dimension that precludes facile layer-by-layer buildup in 3D organ printing. Laser-based printing is also restricted to the processing of low-viscosity materials and requires cells to be immobilized within a gel.

18.1.2 CHALLENGES IN ORGAN PRINTING

Based on a delineation of the aforementioned organ printing techniques and their relative virtues and limitations, it is apparent that significant manufacturing and biological challenges exist and must be overcome for printed organs to be successfully and clinically translated to benefit patients. As might be expected, many of the manufacturing challenges (e.g. resolution enhancement, increasing scalability, and widening manufacturing process windows) are in direct conflict with the biological challenges (e.g. postprint cell viability and functionality). While the incorporation of a vascular network into organs is a significant barrier in tissue engineering, this challenge has been extensively posed and addressed elsewhere, and will not be discussed here.

The authors believe that a key challenge to unlocking the full potential of organ printing is identifying a manufacturing process or perhaps an integration of processes that satisfies two competing requirements: (1) scalability with large-volume patterning for commercial viability with immediate clinical translation; and (2) high-fidelity complex arrangement of cells within extracellular matrices (ECMs) designed to simulate the *in vivo* microstructural niche. On one hand, the former macroscale patterning requirement has been addressed by a category of 3D patterning bioadditive manufacturing processes. Specifically, a number of RP techniques, including microextrusion-based printing, have found significant application in 3D patterning due to their capability to produce scaffold architectures that replicate clinical defect sites and to produce patterned macroscale structures within the bulk construct (Chang et al., 2011; Fedorovich et al., 2011b; Duan and Wang, 2011; Butscher et al., 2011; Kasko and Wong, 2010; Melchels et al., 2010; Jakab et al., 2010). However, this class of RP processes currently lacks the resolution and processing capability needed to meet the design requirement of scaffold feature sizes and geometries characteristic of *in vivo* microstructural cell niches marked by the complex arrangement of cells within ECMs and embedded chemical gradients. These local niches play a pivotal role in the regulation of cellular activity. Therefore, the replication of these structures *in vitro* would allow a more detailed understanding of these microenvironments. On the other hand, the latter resolution requirement has been addressed by processes capable of creating microarchitectures within the required length scale (1–100 μm). In addition to ink-jet-based printing and laser-based printing, this category of techniques that has the resolution required to replicate microstructures also includes microinjection and holographic optical tweezers (Siniscalco et al., 2010; Bible et al., 2009a; Bible et al., 2009b; Masuzaki et al., 2010; Leach et al., 2004; Watson et al., 2004; Akselrod et al., 2006; Townes-Anderson et al., 1998). However, this class of technologies has significant limitations in terms of the maximum areal coverage that can be patterned and the process time needed to complete large-scale 3D fabrication of functional organs. To satisfy these apparently competing design requirements, scalable bioadditive manufacturing processes capable of presenting single-cell level 3D microstructural cues is required. The challenge of creating functional organs with a scalable manufacturing process first requires fundamental understanding of the key polymer rheological material parameters foundational to modeling the bioadditive manufacturing process variables. Although the authors have studied the effects of key process variables in bioadditive manufacturing and engineering 3D cell-based constructs using a microextrusion-based printer and FDM, significant barriers such as microstructural resolution and reproducibility remain and should thus be overcome to enable these bioadditive manufacturing processes to yield functional engineered organs (Chang et al., 2008a; Chang et al., 2008b; Chang et al., 2008c; Chang et al., 2010; Snyder et al., 2011; Buyukhatipoglu et al., 2010; Shor et al., 2009).

Additionally, the authors believe that the full potential of organ printing will necessitate convergence with stem cell technology. For example, mesenchymal stem cells (MSCs) show great promise in tissue engineering applications because of their potential to differentiate into various tissue types, including bone, cartilage, and adipose in response to external cues (Narita et al., 2008; Kim et al., 2009; Delorme et al., 2009; Prockop, 1997). Since MSCs can be isolated from adult patients, this allows for the possibility of using MSCs in regenerative medicine therapies for patient-specific repair of bone and cartilage defects with tissue insensitive to an immune response. Specifically, engineered tissues incorporating MSCs are known to be sensitive to mechanical forces capable of facilitating MSC differentiation into various mature cells (Sikavitsas et al., 2001; Sumanasinghe et al., 2009). For example, cyclic tensile strain and oscillatory fluid flow have both been reported to increase osteogenic differentiation and decrease adipogenic differentiation, whereas uniaxial, unconfined compression and cyclic hydrostatic pressure increase chondrogenesis (Sumanasinghe et al., 2006;

Sumanasinghe et al., 2006; Sen et al., 2008; Arnsdorf et al., 2009; Li et al., 2004; Riddle et al., 2006; Haudenschild et al., 2008; Wagner et al., 2008; Castillo and Jacobs, 2010). A fundamental topic requiring study is the effect of microstructural cues from engineered scaffolds, for instance, the dimensional scale and geometry of the 3D polymer substrates. A number of micro- and nanofabrication-based cell platforms, particularly with spatially controlled features, have been advanced for investigating fundamental questions in cell behavior and tissue development (Whitesides et al., 2001; Théry, 2010; Chen et al., 1997; Quake and Scherer, 2000; Huang et al., 2008). While these micro- and nanoscale manufacturing techniques have yielded insight into structural cues on stem cell differentiation, scalable bioadditive manufacturing processes with embedded microstructural cues for creating functional tissue to meet critical organ deficit size demands have remained unexplored. It has been shown that, given the same material composition, electrospun nanofiber ($<1\ \mu\text{m}$) substrates seeded with MSCs differentiate into bone-forming cells, while solid freeform fabricated ($>100\ \mu\text{m}$) 3D substrates similarly seeded with MSCs yield undifferentiated cells (Farooque et al., 2014; Kumar et al., 2011; Kumar et al., 2012; Huang et al., 2013). Therefore, the author's working hypothesis is that there exists a threshold dimensional scale on the order of the single cell of presenting microstructural cues that will induce MSC differentiation into bone-forming cells. There is a need, therefore, to model and develop enabling bioadditive manufacturing processes to fill in the critical dimensional gap between polymer electrospinning and conventional RP technologies. As a global measure for the process outcome of presenting stem cells with a 3D microstructural niche for differentiation, one possibility is advancing subcellular cell shape dynamics as a quantitative metric. Although there are published reports on single-cell imaging with structural classification of 3D cell shape dimensionality, current limitations of cell shape metrology that need to be addressed include single time point imaging and accounting for the number and distribution of subcellular structural elements (Farooque et al., 2014; Kumar et al., 2011).

18.1.3 MICRO-ORGAN PRINTING AS PHYSIOLOGICAL AND DISEASE PLATFORMS

18.1.3.1 Microextrusion-based Printed Liver Micro-organ on a Chip

A near-future application of organ printing techniques is the creation of cell-based physiological and disease platforms, which we will refer to in this chapter as micro-organ on a chip. Specifically, liver-on-a-chip technology has generated significant research interest in *in vitro* drug metabolism and toxicity studies. However, to date, conventional 2D monolayer cultures of primary hepatocytes cause hepatocytes to lose their morphology and liver-specific functions, including the activity of a group of metabolizing enzymes located in the endoplasmic reticulum called cytochrome P-450 oxidases (Burkhardt et al., 2013). The authors have addressed this obstacle with 3D culture models with layered microextrusion-based printing approaches. Others have also embedded matrices made from natural or synthetic hydrogels and scaffolds constructed from classical biocompatible soft polymers, which enable hepatocytes to express their phenotype in a 3D microenvironment toward increasing their viability and maintaining their functionality over longer culture time periods (Chang et al., 2008a; Chang et al., 2008b; Miranda et al., 2010). It has also been shown that hepatocyte cell physiological behavior is favored under constant perfusion conditions (Goral and Yuen, 2012). In this regard, microfluidics has been adapted through layer-by-layer biofabrication approaches, thus enabling the relatively new "organ-on-a-chip" technology (Lee et al., 2013). This development has led to the creation of micro-analytical micro-organ devices, whose 3D, dynamic nature distinguishes them from the conventional

static *in vitro* models as promising hepatocyte culture testbeds toward preclinical phase testing of drug development (Chang et al., 2008b; Chang et al., 2010).

The authors have implemented a layer-by-layer microextrusion-based printing technique for the creation of cell-encapsulated alginate-based liver micro-organs, which are then integrated onto a microfluidic chamber fabricated using soft lithographic techniques (Chang et al., 2008b; Chang et al., 2010). The system has shown enhanced functionality of HepG2 liver cells within the 3D micro-organ compared to traditional monolayer culture models in terms of cell viability and urea synthesis. Furthermore, the authors have also implemented the microextrusion-based printing in order to construct a cell-encapsulated multilayered tissue construct in a sinusoidal pattern to mimic the *in vivo* liver microarchitecture. The vascularized nature of the liver microenvironment was closely recapitulated by the application of continuous shear-mediated drug perfusion flow through the printed liver micro-organ after its integration within the microfabricated chamber. Drug metabolism studies showed that the system could serve as a reliable 3D *in vitro* pharmacokinetic platform for drug screening and toxicity studies.

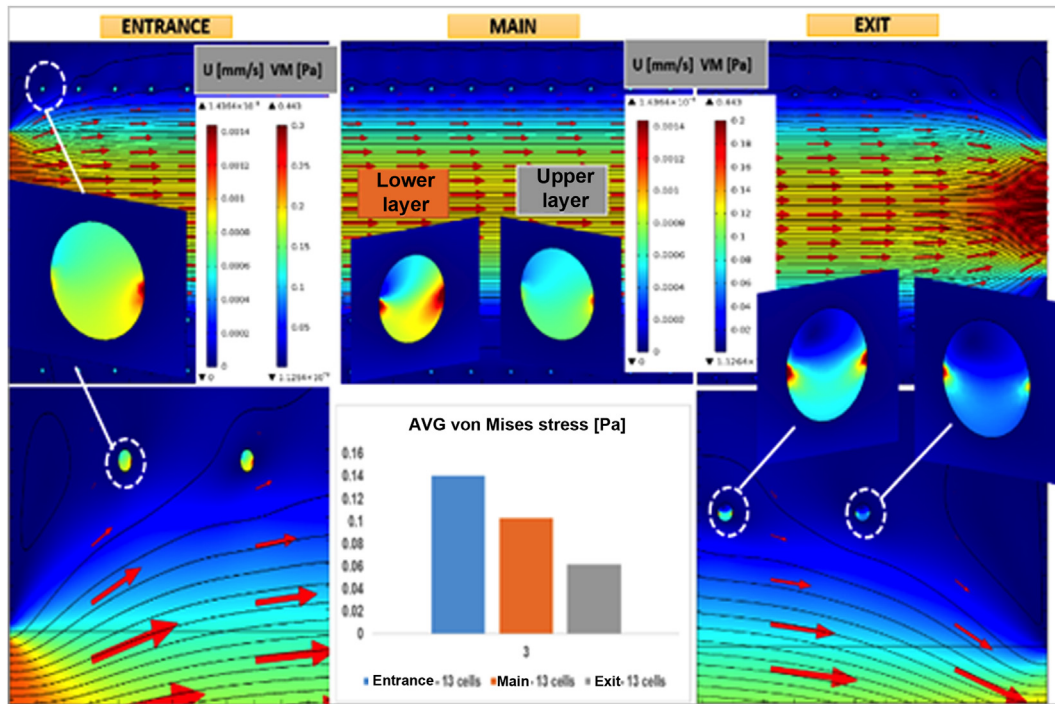
18.1.3.2 Computational Model Setup for Perfused Printed Liver Micro-organ

Intuitively, one can surmise that perfused bioreactors with high cell density, cell encapsulation combined with parallel patterned geometry of biomimetic liver tissue constructs that have been used as microscale drug screening platforms, appear to be a rational approach for drug screening and toxicity studies. However, iterative process design requires advanced computational models that can capture the transport phenomena not only on the macroscale micro-organ level (Tan et al., 2013; Hsu et al., 2014; Huttmacher and Singh, 2008; Truscetto et al., 2011) but also on the single cellular scale. Processing parameters such as flow rate, cell density, and shear stresses on the cell surface need to be correlated with concentration drug profiles and culture time for specific printed liver microarchitectures through appropriate metrics in order to set the design standards that can both predict and promote *in vivo* behavior of encapsulated hepatocytes in dynamic printed micro-organ devices under constant perfusion. The authors have therefore developed a computational modeling strategy for such *in vitro* models using a cell kinetics convection-diffusion problem, where the drug media is convected by the perfusion flow rate from the inlet port of the microscale device, diffused through the hydrogel channel walls due to its inherent porous material nature, and metabolized within the encapsulated hepatocytes.

The steady state results obtained from the simulations done for the free and porous flow regime are presented hereafter. The results obtained by the authors from the transient studies are presented and systematically analyzed elsewhere (Tourlomousis and Chang, November 2014). Using the velocity and the shear stress field computed in steady state, the drug transport and metabolism process is coupled by solving the mass transport equations for the flow and cell domains in time.

18.1.3.3 Steady State Simulations

In the first stage of modeling, the Stokes and Brinkman Equations are solved for an inlet volumetric flow rate of $Q_{in} = 0.25 \mu\text{l/h}$ to ensure a laminar velocity profile across the microfluidic channel as shown in Figure 18.1. The aforementioned value of the inlet volumetric flow rate is prescribed as a starting point for the present study based on the author's experimental drug flow study protocol (Chang et al., 2010). Moreover, the order of magnitude is consistent with values found in previous dynamic bioreactor studies and thus serves as a reasonable starting point. The cells are initially modeled as voids in the computational domain, experience no-slip boundary condition along the walls and thereafter wall shear stress (WSS) as shown in the magnified insets of Figure 18.1. The Newtonian nature of the medium allows the authors to

**FIGURE 18.1**

Velocity field and streamlines indicating a laminar flow topology. Insets: Shear stress field around cells modeled as voids.

compute the WSS as the product of the shear rate with dynamic viscosity around each cell boundary. It is evident from Figure 18.1 that the cell layer exposed directly to the free flow field experiences much higher shear stress compared to the cell layers residing closer to the hydrogel wall. Thus, this part of the study models the cell as being in direct contact with the shear-induced flow field. The average cell WSS range from 0.02 Pa to 0.01 Pa, with maximum WSS values ranging from 0.04 Pa to 0.02 Pa. Based on the geometry model and the cell average WSS computations for all the cells composing the layer of interest, three distinct regimes of the printed micro-organ can be identified in Figure 18.2: (1) the entrance, (2) the middle, and (3) the exit. Each regime contains an equivalent number of cells (13 cells), with the middle cell population experiencing the highest WSS values and the exit cell population experiencing the lowest. Thus, later in the study, one cell from each regime will be considered as representative for the assessment of the steady state results since the distribution of the shear stress magnitude experienced by the cells of each regime is nearly uniform for each regime. By studying only three distinct cells, the authors were able to further downscale the model and thereby evaluate the prototype dynamic micro-organ device.

As a second modeling step, the cells are modeled as linear elastic spheres with a Poisson ratio $\gamma = 0.74$ and elastic modulus $E = 700$ Pa (Wu et al., 2003) under the 2D assumption of plane stress conditions. Average von Mises (VM) stress computations at the cellular level, choosing one cell from each identified regime, reveals, in contrast with the aforementioned cell WSS results, that the entrance cells experience higher VM stresses than the middle cells as shown in Figure 18.3. This can be explained by

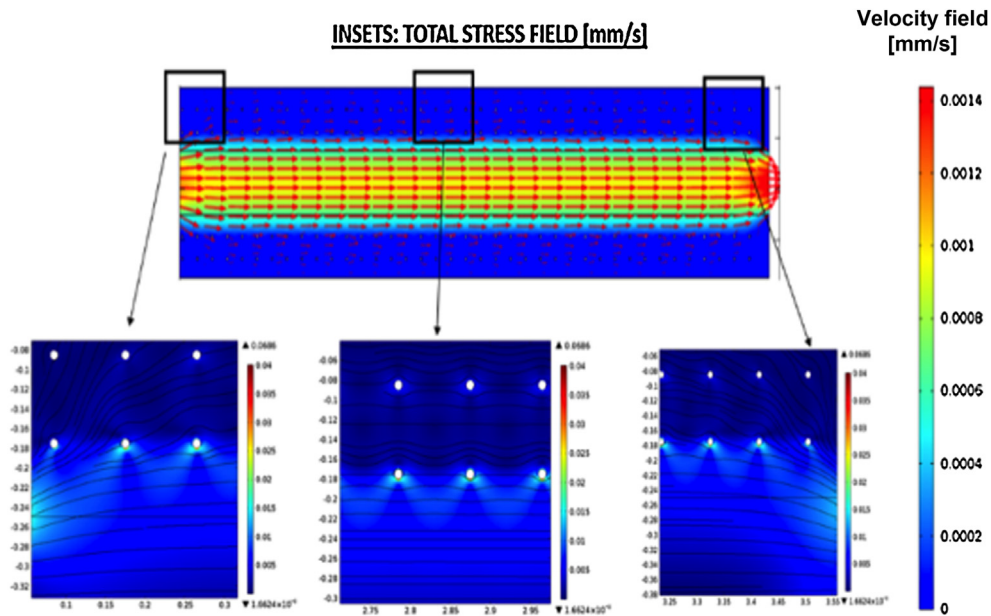


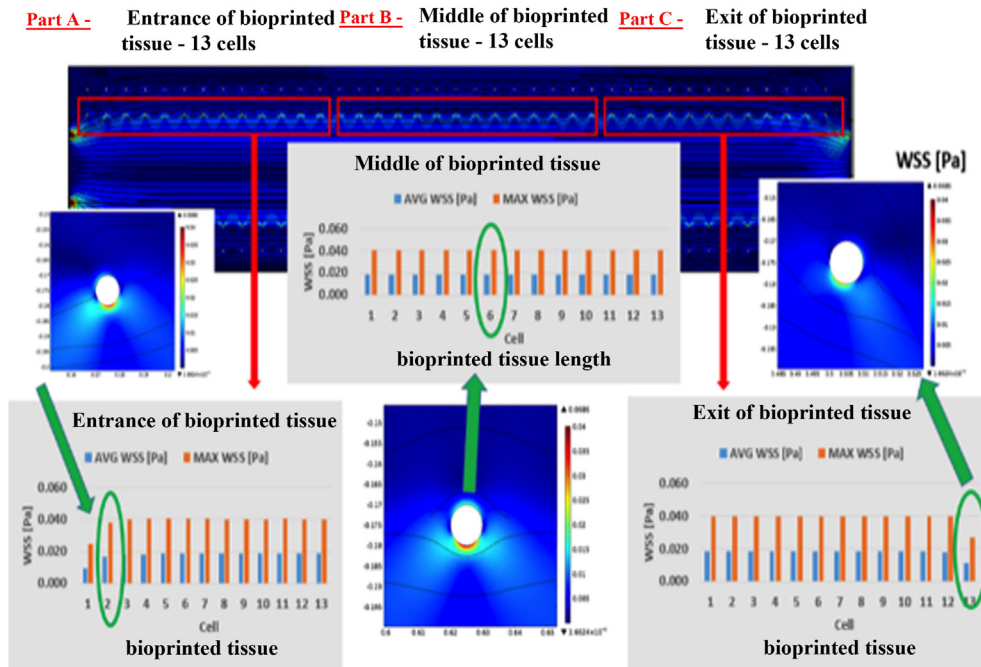
FIGURE 18.2

Identification of three distinct regimes of the printed micro-organ based on average cell WSS and maximum WSS at the single-cell level.

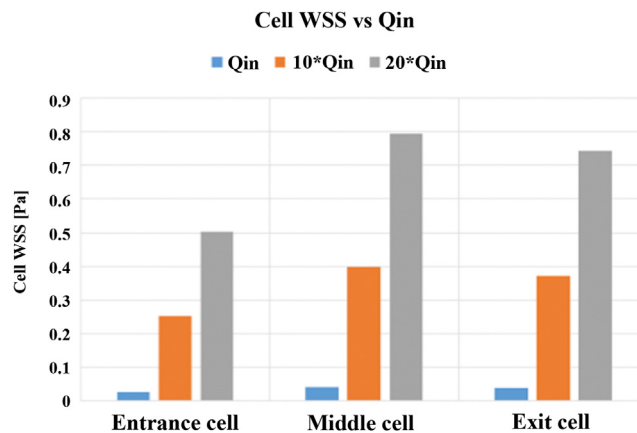
the fact that the VM stress represents the sum of both the normal and shear stresses. Thus, it is evident that the entrance cells experience higher normal stresses compared to the middle cells, which are in contact and in parallel with the fully developed laminar velocity profile of the shear-induced flow field.

However, for the assessment of the dynamic micro-organ device, cell WSS metrics are used, since available literature data for optimum hepatocyte function in dynamic cell cultures is given in terms of WSS. It is reported by Tanaka et al. (Tanaka et al., 2006) that hepatocytes tend to alter their cellular morphology and metabolite function under various conditions of shear-mediated flow in a microchip. In particular, their metabolic activities in terms of albumin synthesis are decreased under shear stresses higher than 2 Pa, increased in the range of 0.14 Pa to 1 Pa and remained relatively constant from 1 to 2 Pa. Moreover, Kan et al. (Kan et al., 1998) tested the shear stress effect on the metabolic function of a coculture system of hepatocytes/parenchymal cells and reported a shear stress value of 0.47 Pa for optimum conditions.

The fact that these reported values are one to two orders of magnitude larger compared to the cell WSS obtained for the dynamic micro-organ device system compelled the authors to vary the magnitude of the inlet volumetric flow rate within the model environment to achieve cell WSS on the same order of magnitude with the results depicted in Figure 18.4. Specifying one cell from each regime of the bioprinted tissue shows that the comparatively low inlet volumetric flow rate in the postprocessing phase needs to be increased 10–20 times from the initial value of 0.25 $\mu\text{L}/\text{h}$ in order for the flow field to induce cell WSS for optimal hepatocyte function.

**FIGURE 18.3**

Velocity field and streamlines indicating a laminar flow topology. Insets: VM stress on each cell modeled as linear elastic spheres. Lower middle: Average VM stress for each regime of the printed micro-organ: (1) entrance, (2) middle, and (3) exit.

**FIGURE 18.4**

Effect of inlet volumetric flow rate on cell WSS.

18.1.4 FUTURE PERSPECTIVES

Based on the existing organ printing techniques and challenges presented in this chapter, the authors foresee the development of an integrated bioadditive manufacturing process to establish smaller feature sizes with prescribed geometries systematically determined by rheological characteristics, process parameters, and machine design. These capabilities will greatly enhance the presentation of precise, tunable microstructural cues in a 3D niche at the single-cell level with concomitant scalable manufacturing of functional organs. In concert with this technology development, also needed is a deeper understanding of biological cell shape metrology in the presence of tunable structural microenvironments or 3D microstructural cell niches for applications in stem cell differentiation and regenerative medicine. Each of these applications requires the creation of engineered 3D functional tissue constructs. To accomplish this, an interdisciplinary research methodology will likely implement engineering model approaches in materials processing, additive manufacturing process modeling, and metrology for benchmarking functional tissue fabrication. Such an endeavor will yield a scalable bioadditive manufacturing process capable of presenting precise, tunable microstructural cues in a 3D niche with single-cell fiber feature sizes and tunable fiber geometries by increasing the control resolution of process parameters for reproducible large-scale fabrication of 3D printed organs. Finally, in the near term, emerging organ printing and microfabrication techniques will converge to enable the direct printing of functional micro-organ-on-a-chip devices. Computational modeling of both printing processes and printed devices (e.g. micro-organ on chip) will be critical in elucidating the mechanically linked biological functional consequences that will further direct future printing process designs.

References

- Ahn, S., Koh, Y. H., & Kim, G. (2010). A three-dimensional hierarchical collagen scaffold fabricated by a combined solid freeform fabrication (SFF) and electrospinning process to enhance mesenchymal stem cell (MSC) proliferation. *Journal of Micromechanics and Microengineering*, 20, 065150.
- Akselrod, G. M., Timp, W., Mirsaidov, U., Zhao, Q., Li, C., Timp, R., Timp, K., Matsudaira, P., & Timp, G. (2006). Laser-guided assembly of heterotypic three-dimensional living cell microarrays. *Biophysical Journal*, 91, 3465.
- Arnsdorf, E. J., Tummala, P., Kwon, R. Y., & Jacobs, C. R. (2009). Mechanically induced osteogenic differentiation: the role of RhoA, ROCKII, and cytoskeletal dynamics. *Journal of Cell Science*, 122, 546.
- Bible, E., Chau, D. Y. S., Alexander, M. R., Price, J., Shakesheff, K. M., & Modo, M. (2009b). Attachment of stem cells to scaffold particles for intracerebral transplantation. *Nature Protocols*, 4, 1440.
- Bible, E., Chau, D. Y. S., Alexander, M. R., Price, J., Shakesheff, K. M., & Modo, M. (2009a). The support of neural stem cells transplanted into stroke-induced brain cavities by PLGA particles. *Biomaterials*, 30, 2985.
- Boland, T., Xu, T., Damon, B., & Cui, X. (2006). Application of inkjet printing to tissue engineering. *Biotechnology Journal*, 1(9), 910.
- Burdick, J. A., & Vunjak-Novakovic, G. (2009). Engineered microenvironments for controlled stem cell differentiation. *Tissue Engineering Part A*, 15(2), 205.
- Burkhardt, B., Martinez-Sanchez, J. J., Bachmann, A., Ladurner, R., & Nüssler, A. K. (2013). Long-term culture of primary hepatocytes: new matrices and microfluidic devices. *Hepatology International*, 8(1), 14.
- Butscher, A., Bohner, M., Hofmann, S., Gauckler, L., & Müller, R. (2011). Structural and material approaches to bone tissue engineering in powder-based three-dimensional printing. *Acta Biomaterials*, 7, 907.
- Buyukhatipoglu, K., Chang, R., Sun, W., & Morss-Clyne, A. (2010). Bioprinted nanoparticles for tissue engineering applications. *Tissue Engineering Part C*, 4(16), 631.

- Castillo, A. B., & Jacobs, C. R. (2010). Mesenchymal stem cell mechanobiology. *Current Osteoporosis Reports*, 8(2), 98.
- Chang, C. C., Boland, E. D., Williams, S. K., & Hoying, J. B. (2011). Direct-write bioprinting three-dimensional biohybrid systems for future regenerative therapies. *Journal of Biomedical Materials Research B Applied Biomaterials*, 98B, 160.
- Chang, R., Emami, K., Wu, H., & Sun, W. (2010). Biofabrication of a three-dimensional liver micro-organ as an *in vitro* drug metabolism model. *Biofabrication*, 2(4), 045004.
- Chang, R., Nam, J., & Sun, W. (2008c). Computer-Aided Design, Modeling, and Freeform Fabrication of Micro-organ Flow Patterns for Pharmacokinetic Study. *CAD Applications*, 5, 21.
- Chang, R., Nam, J., & Sun, W. (2008b). Direct cell writing of 3D micro-organ for *in vitro* pharmacokinetic model. *Tissue Engineering Part C*, 2(14), 157.
- Chang, R., Nam, J., & Sun, W. (2008a). Effects of dispensing pressure and nozzle diameter on cell survival from solid freeform fabrication-based direct cell writing. *Tissue Engineering*, 1(14), 41.
- Chen, C. S., Mrksich, M., Huang, S., Whitesides, G. M., & Ingber, D. E. (1997). Geometric control of cell life and death. *Science*, 276(5317), 1425.
- Cohen, D. L., Malone, E., Lipson, H., & Bonassar, L. J. (2009). Direct freeform fabrication of seeded hydrogels in arbitrary geometries. *Tissue Engineering*, 12(5), 1325.
- Delorme, B., Ringe, J., Pontikoglou, C., Gaillard, J., Langornné, A., Sensebé, L., Noël, D., Jorgensen, C., Häupi, T., & Charbord, P. (2009). Specific lineage-priming of bone marrow mesenchymal stem cells provides the molecular framework for their plasticity. *Stem Cells*, 27(5), 1142.
- Duan, B., & Wang, M. (2011). Selective laser sintering and its application in biomedical engineering. *Materials Research Society Bulletin*, 36, 998.
- Farooque, T. M., Camp, C. H., Tison, C. K., Kumar, G., Parekh, S. H., & Simon, C. G. (2014). Measuring stem cell dimensionality in tissue scaffolds. *Biomaterials*, 35(9), 2558.
- Fedorovich, N. E., Alblas, J., Hennink, W. E., Oner, F. C., & Dhert, W. J. A. (2011b). Organ printing: the future of bone regeneration? *Trends in Biotechnology*, 29, 601.
- Fedorovich, N. E., Dewijn, J. R., Verbout, A. J., Alblas, J., & Dhert, W. J. (2008). Three-dimensional fiber deposition of cell-laden, viable, patterned constructs for bone tissue printing. *Tissue Engineering Part A*, 14(1), 127.
- Fedorovich, N. E., Kuipers, E., Gawlitta, D., Dhert, W. J., & Alblas, J. (2011a). Scaffold porosity and oxygenation of printed hydrogel constructs affect functionality of embedded osteogenic progenitors. *Tissue Engineering Part A*, 17(19), 2473.
- Gaetani, R., Doevendans, P. A., Metz, C. H., Alblas, J., Messina, E., Giacomello, A., & Sluijter, J. P. (2012). Cardiac tissue engineering using tissue printing technology and human cardiac progenitor cells. *Biomaterials*, 33(6), 1782.
- Goral, V. N., & Yuen, P. K. (2012). Microfluidic platforms for hepatocyte cell culture: new technologies and applications. *Annals of Biomedical Engineering*, 40(6), 1244.
- Gruene, M., Pflaum, M., Hess, C., Diamantouros, S., Schlie, S., Deiwick, A., Koch, L., Wilhelmi, M., Jockenhoevel, S., Haverich, A., & Chichkov, B. (2011). Laser printing of three-dimensional multicellular arrays for studies of cell-cell and cell-environment interactions. *Tissue Engineering Part C*, 17(10), 973.
- Guillemot, F., Souquet, A., Castros, S., & Guillotin, B. (2010). Laser-assisted cell printing: principle, physical parameters versus cell fate and perspectives in tissue engineering. *Nanomedicine*, 5(3), 507.
- Haudenschild, A. K., Hsieh, A. H., Kapila, S., & Lotz, J. C. (2008). Pressure and distortion regulate human mesenchymal stem cell gene expression. *Annals of Biomedical Engineering*, 37(3), 492.
- Hsu, M. N., Tan, G. D., Tania, M., Birgersson, E., & Leo, H. L. (2014). Computational fluid model incorporating liver metabolic activities in perfusion bioreactor. *Biotechnology and Bioengineering*, 111(5), 885.
- Huang, A. H., Motlekar, N. A., Stein, A., Diamond, S. L., Shore, E. M., & Mauck, R. L. (2008). High-throughput screening for modulators of mesenchymal stem cell chondrogenesis. *Annals of Biomedical Engineering*, 36(11), 1909.
- Huang, G. S., Tseng, C. S., Yen, B., Linju, Hsieh, P. S., & Hsu, S. H. (2013). Solid freeform-fabricated scaffolds designed to carry multicellular mesenchymal stem cell spheroids for cartilage regeneration. *European Cells and Materials*, 26, 179.

- Hutmacher, D. W., & Singh, H. (2008). Computational fluid dynamics for improved bioreactor design and 3D culture. *Trends in Biotechnology*, 26(4), 166.
- Jakab, K., Norotte, C., Marga, F., Murphy, K., Vunjak-Novakovic, G., & Forgacs, G. (2010). Tissue engineering by self-assembly and bioprinting of living cells. *Biofabrication*, 2, 022001.
- Kan, P., Miyoshi, H., Yanagi, K., & Ohshima, N. (1998). Effects of shear stress on metabolic function of the coculture system of hepatocyte/nonparenchymal cells for a bioartificial liver. *ASAIO Journal*, 44(5), M441.
- Kasko, A. M., & Wong, D. Y. (2010). Two-photon lithography in the future of cell-based therapeutics and regenerative medicine: a review of techniques for hydrogel patterning and controlled release. *Future Medicinal Chemistry*, 2, 1669.
- Khalil, S., & Sun, W. (2009). Bioprinting endothelial cells with alginate for 3D tissue constructs. *Journal of Biomechanical Engineering*, 131(11), 111002.
- Kim, M. R., Jeon, E. S., Kim, M. Y., Lee, J. S., & Kim, J. H. (2009). Thromboxane a₂ induces differentiation of human mesenchymal stem cells to smooth muscle-like cells. *Stem Cells*, 27(1), 191.
- Koch, L., Kuhn, S., Sorg, H., Gruene, M., Schlie, S., Gaebel, R., Polchow, B., Reimers, K., Stoelting, S., Ma, N., Vogt, P. M., Steinhoff, G., & Chichkov, B. (2010). Laser printing of skin cells and human stem cells. *Tissue Engineering Part C*, 16(5), 847.
- Kumar, G., Tison, C. K., Chatterjee, K., Pine, P. S., McDaniel, J. H., Salit, M. L., & Simon, C. G. (2011). The determination of stem cell fate by 3D scaffold structures through the control of cell shape. *Biomaterials*, 32(35), 9188.
- Kumar, G., Waters, M. S., Farooque, T. M., Young, M. F., & Simon, C. G. (2012). Freeform fabricated scaffolds with roughened struts that enhance both stem cell proliferation and differentiation by controlling cell shape. *Biomaterials*, 33(16), 4022.
- Leach, J., Sinclair, G., Jordan, P., Courtial, J., Padgett, M. J., Cooper, J., & Laczik, Z. (2004). 3D manipulation of particles into crystal structures using holographic optical tweezers. *Optics Express*, 12, 220.
- Lee, J., Kim, S. H., Kim, Y. C., Choi, I., & Sung, J. H. (2013). Fabrication and characterization of microfluidic liver-on-a-chip using microsomal enzymes. *Enzyme and Microbial Technology*, 53(3), 159.
- Li, M., Tian, X., Schreyer, D. J., & Chen, X. (2011). Effect of needle geometry on flow rate and cell damage in the dispensing-based biofabrication process. *Biotechnology Progress*, 27(6), 1777.
- Li, Y. J., Batra, N. N., You, L., Meier, S. C., Coe, I. A., Yellowley, C. E., & Jacobs, C. R. (2004). Oscillatory fluid flow affects human marrow stromal cell proliferation and differentiation. *Journal of Orthopedic Research*, 22(6), 1283.
- Maher, P. S., Keatch, R. P., Donnelly, K., Mackay, R. E., & Paxton, J. Z. (2009). Construction of 3D biological matrices using rapid prototyping technology. *Rapid Prototyping Journal*, 15(3), 204.
- Masuzaki, T., Ayukawa, Y., Moriyama, Y., Jinno, Y., Atsuta, I., Ogino, Y., & Koyano, K. (2010). The effect of a single remote injection of statin-impregnated poly (lactic-co-glycolic acid) microspheres on osteogenesis around titanium implants in rat tibia. *Biomaterials*, 31, 3327.
- Melchels, F. P. W., Domingos, M. A. N., Klein, T. J., Malda, J., Bartolo, P. J., & Hutmacher, D. W. (2012). Additive manufacturing of tissues and organs. *Progress in Polymer Science*, 37(8), 1079.
- Melchels, F. P. W., Feijen, J., & Grijpma, D. W. (2010). A review on stereolithography and its applications in biomedical engineering. *Biomaterials*, 31, 6121.
- Mezel, C., Souquet, A., Hallo, L., & Guillemot, F. (2010). Bioprinting by laser-induced forward transfer for tissue engineering applications: jet formation modeling. *Biofabrication*, 2(1), 014103.
- Miranda, J. P., Rodrigues, A., Tostoes, R. M., Leite, S., Zimmerman, H., Carrondo, M. J., & Alves, P. M. (2010). Extending hepatocyte functionality for drug-testing applications using high-viscosity alginate-encapsulated three-dimensional cultures in bioreactors. *Tissue Engineering Part C*, 16(6), 1223–1232.
- Mironov, V., Boland, T., Trusk, T., Forgacs, G., & Markwald, R. R. (2003). Organ printing: computer-aided jet-based 3D tissue engineering. *Trends in Biotechnology*, 21(4), 157.
- Mironov, V., Kasyanov, V., Drake, C., & Markwald, R. R. (2008). Organ printing: promises and challenges. *Regenerative Medicine*, 3(1), 93.
- Mironov, V., Kasyanov, V., & Markwald, R. R. (2011). Organ printing: from bioprinter to organ biofabrication line. *Current Opinions in Biotechnology*, 22(5), 667.

- Mironov, V., Visconti, R. P., Kasyanov, V., Forgacs, G., Drake, C. J., & Markwald, R. R. (2009). Organ printing: tissue spheroids as building blocks. *Biomaterials*, 30(12), 2164.
- Nakamura, M., Iwanaga, S., Henmi, C., Arai, K., & Nishiyama, Y. (2010). Biomatrices and biomaterials for future developments of bioprinting and biofabrication. *Biofabrication*, 2(1), 014110.
- Narita, Y., Yamawaki, A., Kagami, H., Ueda, M., & Ueda, Y. (2008). Effects of transforming growth factor-beta 1 and ascorbic acid on differentiation of human bone-marrow-derived mesenchymal stem cells into smooth muscle cell lineage. *Cell and Tissue Research*, 333(3), 449.
- Nishiyama, Y., Nakamura, M., Henmi, C., Yamaguchi, K., Mochizuki, S., Nakagawa, H., & Takiura, K. (2009). Development of a three-dimensional bioprinter: construction of cell-supporting structures using hydrogel and state-of-the-art inkjet technology. *Journal of Biomechanical Engineering*, 131(3), 035001.
- Prockop, D. J. (1997). Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science*, 276(5309), 71.
- Quake, S. R., & Scherer, A. (2000). From micro- to nanofabrication with soft materials. *Science*, 290(5496), 1536.
- Rezende, R. A., Pereira, F. D. A. S., Kasyanov, V., Kemmoku, D. T., Maia, I., da Silva, J. V. L., & Mironov, V. (2013). Scalable biofabrication of tissue spheroids for organ printing. *Procedia CIRP*, 5, 276.
- Riddle, R. C., Taylor, A. F., Genetos, D. C., & Donahue, H. J. (2006). MAP kinase and calcium signaling mediate fluid flow-induced human mesenchymal stem cell proliferation. *American Journal of Physiology and Cell Physiology*, 290(3), C776.
- Roth, E. A., Xu, T., Das, M., Gregory, C., Hickman, J. J., & Boland, T. (2004). Inkjet printing for high-throughput cell patterning. *Biomaterials*, 25(17), 3707.
- Sekula, S., Fuchs, J., Weg-Remers, S., Nagel, P., Schupler, S., Fragala, J., Theilacker, N., Franzreb, M., Wingren, C., Ellmark, P., Borrebaeck, C. A., Mirkin, C. A., Fuchs, H., & Lenhart, S. (2008). Multiplexed lipid dip-pen nanolithography on subcellular scales for the templating of functional proteins and cell culture. *Small*, 4(10), 1785.
- Sen, B., Xie, Z., Case, N., Ma, M., Rubin, C., & Rubin, J. (2008). Mechanical strain inhibits adipogenesis in mesenchymal stem cells by stimulating a durable beta-catenin signal. *Endocrinology*, 149(12), 6065.
- Shim, J. H., Kim, J. Y., Park, M., Park, J., & Cho, D. W. (2011). Development of a hybrid scaffold with synthetic biomaterials and hydrogel using solid freeform fabrication technology. *Biofabrication*, 3(3), 034102.
- Shor, L., Guceri, S., Chang, R., Gordon, J., Kang, Q., Hartstock, L., An, Y., & Sun, W. (2009). Precision extruding deposition (PED) fabrication of poly-ε-caprolactone (PCL) scaffolds for bone tissue engineering. *Biofabrication*, 1(1), 015003.
- Sikavitsas, V. I., Temenoff, J. S., & Mikos, A. G. (2001). Biomaterials and bone mechanotransduction. *Biomaterials*, 22(19), 2581.
- Siniscalco, D., Giordano, C., Galderisi, U., Luongo, L., Alessio, N., Di Bernardo, G., de Novellis, V., Rossi, F., & Malone, S. (2010). Intrabrain microinjection of human mesenchymal stem cells decreases allodynia in neuropathic mice. *Cellular and Molecular Life Sciences*, 67, 655.
- Smith, C. M., Christian, J. J., Warren, W. L., & Williams, S. K. (2007). Characterizing environmental factors that impact the viability of tissue-engineered constructs fabricated by a direct-write bioassembly tool. *Tissue Engineering*, 13(2), 373.
- Smith, C. M., Stone, A. L., Parkhill, R. L., Stewart, R. L., Simpkins, M. W., Kachurin, A. M., Warren, W. L., & Williams, S. K. (2004). Three-dimensional bioassembly tool for generating viable tissue-engineered constructs. *Tissue Engineering*, 10(9), 1566.
- Snyder, J., Hamid, Q., Wang, W., Chang, R., Emami, K., Wu, H., & Sun, W. (2011). Bioprinting cell-laden matrigel for radioprotection study of liver by prodrug conversion in dual tissue microfluidic chip. *Biofabrication*, 3(3), 034112.
- Stringer, J., & Derby, B. (2010). Formation and stability of lines produced by inkjet printing. *Langmuir*, 26(12), 10365.
- Sumanasinghe, R. D., Bernacki, S. H., & Lobo, E. G. (2006). Osteogenic differentiation of human mesenchymal stem cells in collagen matrices: effect of uniaxial cyclic tensile strain on bone morphogenetic protein (BMP-2) mRNA expression. *Tissue Engineering*, 12(12), 3459.
- Sumanasinghe, R. D., Pfeiler, T. W., Monteiro-Riviere, N. A., & Lobo, E. G. (2009). Expression of proinflammatory cytokines by human mesenchymal stem cells in response to cyclic tensile strain. *Journal of Cell Physiology*, 219(1), 77.

- Tan, G. D., Toh, G. W., Birgersson, E., Robens, J., van Noort, D., & Leo, H. L. (2013). A thin-walled polydimethylsiloxane bioreactor for high-density hepatocyte sandwich culture. *Biotechnology and Bioengineering*, 110(6), 1663.
- Tanaka, Y., Yamato, M., Okano, T., Kitamori, T., & Sato, K. (2006). Evaluation of effects of shear stress on hepatocytes by a microchip-based system. *Measurement Science and Technology*, 17, 3167.
- Théry, M. (2010). Micropatterning as a tool to decipher cell morphogenesis and functions. *Journal of Cell Science*, 123, 4201.
- Tourlomis, F., & Chang, R. C. (November 2014). *Computational modeling of 3D printed tissue-on-a-chip microfluidic devices as drug screening platforms*. Montreal, Canada: Proceedings of ASME, IMECE.
- Townes-Anderson, E., St Jules, R. S., Sherry, D. M., Lichtenberger, J., & Hassanain, M. (1998). Micromanipulation of retinal neurons by optical tweezers. *Molecular Vision*, 4, 12.
- Truscello, S., Schrooten, J., & Van, H. Oosterwyck (2011). A computational tool for the upscaling of regular scaffolds during *in vitro* perfusion culture. *Tissue Engineering Part C*, 17(6), 619.
- Wagner, D. R., Lindsey, D. P., Li, K. W., Tummala, P., Chandran, S. E., Smith, R. L., Longaker, M. T., Carter, D. R., & Beaupre, G. S. (2008). Hydrostatic pressure enhances chondrogenic differentiation of human bone marrow stromal cells in osteochondrogenic medium. *Annals of Biomedical Engineering*, 36(5), 813.
- Wang, X., Yan, Y., Pan, Y., Xiong, Z., Liu, H., Cheng, J., Liu, F., Wu, R., Zhang, R., & Lu, Q. (2006). Generation of three-dimensional hepatocyte/gelatin structures with rapid prototyping system. *Tissue Engineering*, 12(1), 83.
- Watson, D., Hagen, N., Diver, J., Marchand, P., & Chachisvilis, M. (2004). Elastic light scattering from single cells: orientational dynamics in optical trap. *Biophysical Journal*, 87, 1298.
- Whitesides, G. M. (1995). Self-assembling materials. *Scientific American*, 273(3), 114.
- Whitesides, G. M., & Grzybowski, B. (2002). Self-Assembly at all scales. *Science*, 295(5564), 2418.
- Whitesides, G. M., Ostuni, E., Takayama, S., Jiang, X., & Ingber, D. E. (2001). Soft lithography in biology and biochemistry. *Annual Review of Biomedical Engineering*, 3, 335–373.
- Wu, Z. Z., Zhang, G., Long, M., Wang, H. B., Song, G. B., & Cai, S. X. (2003). Comparison of the viscoelastic properties of normal hepatocytes and hepatocellular carcinoma cells under cytoskeletal perturbation. *Biorheology*, 37(4), 279.
- Xu, T., Jin, J., Gregory, C., Hickman, J. J., & Boland, T. (2005). Inkjet printing of viable mammalian cells. *Biomaterials*, 26(1), 93.
- Yan, Y., Wang, X., Pan, Y., Liu, H., Cheng, J., Xiong, Z., Lin, F., Wu, R., Zhang, R., & Lu, Q. (2005). Fabrication of viable tissue-engineered constructs with 3D cell-assembly technique. *Biomaterials*, 26(29), 5864.

3D Bioprinting, Nanotechnology, and Intellectual Property

19

Elizabeth D. Ferrill and Kai Rajan

Finnegan, Henderson, Farabow, Garrett & Dunner, LLP, Washington, DC, United States

19.1 INTRODUCTION

Like many modern technologies, 3D printing and nanotechnology are improving at breakneck speed, through innovations and improvements in machines, software, methods, and materials. These innovations and improvements are made possible by inventors and researchers striving to benefit society. In addition to the satisfaction of benefitting society, the inventors and researchers, as well as the companies and institutions that fund and enable research, need assurance that the time and money spent advancing technology can be recovered by successful innovations, to fund additional research and maintain the cycle of research and innovation. Intellectual property (IP) laws provide this assurance.

IP broadly refers to “creations of the mind, such as inventions; literary and artistic works; designs; and symbols, names, and images used in commerce.”² IP arises from laws that protect these creations of the mind from unfair exploitation, including laws for patents, copyrights, trade secrets, and trademarks. IP enables inventors and creators to protect their financial investments in research by securing the profits from successful and popular inventions or creations. In return, the public is enriched by inventions and creations. By striking the right balance between protecting the interests of innovators and the public, IP laws aim to foster an environment in which creativity and innovation can flourish.

IP laws aim to benefit society and reward innovators, but there are exceptions and limitations to protection, and there are challenges to enforcing IP rights. This chapter explores the different types of IP, how they protect 3D bioprinting and nanotechnology (particularly engineered tissue), limitations on IP, and how IP owners protect and enforce their rights.

19.2 WHY IS INTELLECTUAL PROPERTY IMPORTANT?

IP laws foster advancements in science by providing mechanisms to (1) protect research and innovation from copycats, thieves, and even accidental copying and (2) provide recourse for IP owners. Such

¹Elizabeth Ferrill is a partner and Kai Rajan is an associate with the law firm of Finnegan, Henderson, Farabow, Garrett & Dunner, LLP, based in Washington, DC, <http://www.finnegan.com>. Special thanks goes to John Hornick, a retired partner of the Finnegan law firm, for his invaluable assistance with the first edition of this chapter. John was a frequent writer and lecturer on IP and 3D printing, educating and advising clients in this space. Any opinions expressed in this chapter are those of the authors, not of Finnegan. This chapter does not provide legal advice.

²World Intellectual Property Organization, *What is Intellectual Property?* <http://www.wipo.int/about-ip/en/>.

protections and rights incentivize innovators and investors to spend time and money to develop innovations and improvements in products and services that benefit society. Patent protection particularly enables inventors and investors to capitalize on their investments in research and development (R&D) by deterring or slowing down competitors and forcing them to innovate in different ways to avoid infringing patents.³

The ability to protect IP is especially important in the medical industry. Anything that is used on or in the human body requires extensive testing to ensure biocompatibility and to evaluate side effects.⁴ In the United States, medical devices, drugs, and implants are evaluated and approved by submitting volumes of testing data to a regulatory agency: the Food and Drug Administration (FDA). The FDA continuously monitors new and developing technologies, such as 3D bioprinting, and provides guidance as to the types of testing required to evaluate new products such as printed tissue.⁵ Such testing substantially raises the costs of R&D for new products such as drugs and implants. Innovators experimenting with new compounds, material configurations, products, and methods bear the burden of research costs, with the hope that some products will be successful, receive approval for sale, generate enough revenue to recoup R&D costs for both successful and unsuccessful products, and make a profit.

R&D costs in medical technologies can be prohibitively expensive, causing some companies to copy other successful products and sell them as their own. This creates unhealthy competition that deprives innovators and investors of profits and returns on their investments, ultimately discouraging further R&D for fear of copying. Indeed, high R&D costs coupled with lost profits from copycatting can demoralize research initiatives and stifle innovation. IP helps to prevent copycats from profiting from free rides on others' R&D and promotes healthy competition.

Our modern IP system fosters innovation, but the system and its laws are not without controversy and complications. New and emerging technologies that do not fit squarely into current IP laws cause confusion and inconsistency in IP protection. For example, in recent decades, inventors, their attorneys, the courts, and even Congress have struggled with the patentability of software, because the original patent laws and even recent amendments do not explicitly address it.⁶ Similarly, despite their potential to advance science by leaps and bounds, 3D bioprinting technology, nanotechnology, and tissue engineering technology raise more questions about what aspects of these new inventions and improvements *can* be protected by IP, which *cannot* be protected, and which *should not* be protected for ethical and public policy reasons.

³Kapmar, D. (2013). *A Look At The Patentability Of 3-D Printed Human Organs*, Published May 28, 2013, <http://www.law360.com/articles/439549/a-look-at-the-patentability-of-3-d-printed-human-organs>.

⁴United States Food and Drug Administration. (2012). *Medical Devices: PMA Application Methods*, Last updated February 14, 2012, <http://www.fda.gov/medicaldevices/deviceregulationandguidance/howtomarketyourdevice/premarket-submissions/premarketapprovalpma/ucm048168.htm>; United States Food and Drug Administration, *Vaccines, Blood, and Biologics: Tissue and Tissue Product Questions and Answers*, Updated October 14, 2009, <http://www.fda.gov/BiologicsBloodVaccines/TissueTissueProducts/QuestionsaboutTissues/ucm101559.htm>.

⁵United States Food and Drug Administration. (2017). *Statement by FDA Commissioner Scott Gottlieb, M.D., on FDA ushering in new era of 3D printing of medical products; provides guidance to manufacturers of medical devices*, Updated December 4, 2017, <https://www.fda.gov/news-events/press-announcements/statement-fda-commissioner-scott-gottlieb-md-fda-ushering-new-era-3d-printing-medical-products>.

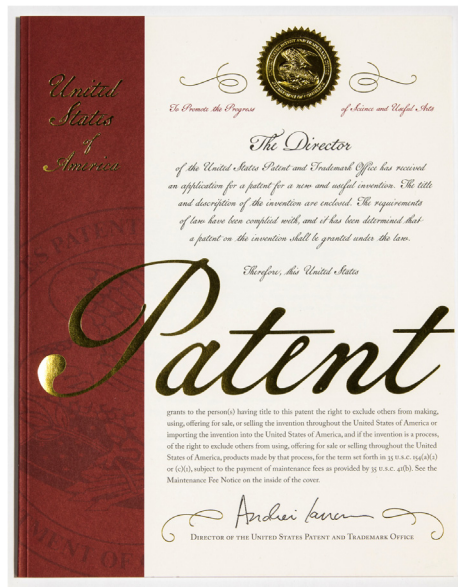
⁶*Alice Corp. v. CLS Bank Int'l*, 573 United States 208, 134S. Ct. 2347 (2014); *Gottschalk v. Benson*, 409 United States 63 (1972); *Parker v. Flook*, 437 United States 584 (1978); *Diamond v. Diehr*, 450 United States 175 (1981); *Bilski v. Kappos*, 561 US 593, 130S. Ct. 3218 (2010).

19.3 TYPES OF INTELLECTUAL PROPERTY

IP laws are national systems. This chapter focuses on the US IP laws. The US IP laws protect innovations and creations through patents, trademarks, copyrights, and trade secrets. However, IP protection in the United States does not extend throughout the world. Rather, with the exception of the European Union, inventors must seek IP protection in each country where protection is desired, either through filing separate applications or complying with national laws. Although IP laws vary by country, the types of IP protection available throughout the world are fairly consistent.

19.3.1 PATENTS

Patents provide inventors of new and useful inventions the right “to exclude others from making, using, offering for sale, or selling the invention throughout the United States or importing the invention into the United States.”⁷ This right lasts up to 20 years in the United States. There are three types of patents: utility patents; design patents; and plant patents. *Utility* patents protect new and useful machines, processes, compositions of matter, and articles of manufacture. Utility patents can also protect computer programs and software, by describing the functions performed by a computer. *Design* patents protect the decorative look (called the “ornamental design”) of objects. Design patents also protect the appearance of computer icons, graphic user interfaces, and graphic animations. Finally, *plant* patents protect newly invented or discovered plant varieties.



⁷United States Patent & Trademark Office. *What is a Patent?* <http://www.uspto.gov/patents/>.

To obtain a patent, the inventor(s) must disclose the details of their invention to the US Patent Office in a patent application, which is examined by a patent examiner to determine whether the invention will be patented.⁸ The examination process can take years, but IP protection for granted patents reaches back to the date the patent application was filed. Once a patent is granted, the details of the invention are released to the public, to increase public knowledge, and to inform the public of the inventors' exclusive rights. In return for enhancing the public knowledge, the inventors are granted the exclusive right to make, use, and sell the invention for 20 years from the filing date.

After obtaining a patent, the patent owner may choose to sell the patent, grant licenses to others who want to make, use, or sell the patented invention for limited periods of time or enforce patent rights against infringers making, using, and/or selling the same invention without authorization. Notably, infringers can be liable to the patent owner even if they did not know the patent existed, because independent discovery is not an excuse or defense to patent infringement. Patent infringement and enforcing IP rights for 3D bioprinting and nanotechnology are addressed later in this chapter.

19.3.2 COPYRIGHTS

Copyrights protect original creative works of authorship, such as writing, music, and even software. Copyright protection for software covers the particular code written for a computer program, in contrast to software patents that protect the functionality that the software provides when the software is run on a computer. A copyright provides the author of the creative work with the exclusive right to distribute and use that creative work and prevents others from copying the creative work for up to 70 years after the author's death.⁹ Although this may seem like a very long time, others can often avoid copyright infringement by interpreting work in their own creative way, such as by rewriting work in their own words, or developing equivalent software in a different style of coding. Thus copyright protection primarily prevents someone from simply copying the creative work of another and claiming the copied creativity as his or her own.

Copyright protection arises automatically when the work is fixed in a tangible form. Authors may also register their creative works with the US Copyright Office and can display the "©" symbol at any time. Copyright owners can sell their rights, grant licenses to others to use or reproduce the copyrighted work, and sue copycats who reproduce the copyrighted work. Granting a copyright license can be as simple as purchasing a computer program because authorized sales of such works often include licenses to use (and sometimes reproduce) the copyrighted work.

⁸United States Patent and Trademark Office, *Process for Obtaining a Utility Patent*, <http://www.uspto.gov/patents/process/index.jsp>.

⁹United States Copyright Office. (2021). *What is Copyright?*, Updated February 2021, <https://www.copyright.gov/what-is-copyright/>.



19.3.3 TRADEMARKS

Trademarks are words, phrases, symbols, and designs that identify the source of goods or services.¹⁰ Trademarks protect manufacturers, merchants, and consumers by preventing impostors from posing as reputable companies with successful products and services. Unlike patents, trademarks do not need to be registered with the US Patent and Trademark Office, although formal registration provides additional layers of protection. Registered trademarks are distinguished by the ® symbol. Trademark protection can cover unregistered trademarks after an individual or company that owns the trademark starts using the mark in commerce, and once the trademark gains recognition and association with the trademark owner's goods or services. Unregistered trademarks for products can bear the ™ symbol, and unregistered service marks for services can bear the ℠ symbol.¹¹



To register a trademark, the owner must submit the mark to the US Patent and Trademark Office, along with proof that the owner intends to use or is currently using the mark in commerce. If the mark is not already in use by another and is not too similar to other marks, it will most likely be registered. The registration process is generally much quicker than the examination procedure for patents. Those who do not wish to register their marks can use the ™ and ℠ notations at any time. However, trademark registration provides the owner of the mark with increased rights and options for stopping infringers. For example, registering a trademark lets the public know that a particular trademark is “taken,” and that copycats will be liable for trademark infringement.¹²

¹⁰United States Patent and Trademark Office. (2021). *What is a trademark?*, Updated January 2021, <https://www.uspto.gov/trademarks/basics/trademark-patent-or-copyright>

¹¹United States Patent and Trademark Office, *Trademark, Patent, or Copyright?* Updated January 18, 2013, <http://www.uspto.gov/trademarks/basics/definitions.jsp>.

¹²United States Patent and Trademark Office. (2013). *Trademarks FAQs: What are the benefits of federal trademark registration?* Updated April 23, 2013, http://www.uspto.gov/faq/trademarks.jsp#_Toc275426681.

Trademarks are mainly used for company and product branding. In tissue engineering, the products are tissue and organs. While implanted devices often have some type of marking or model identifier, printed tissue and organs may not carry logos or branding, but time will tell. At this time, trademarks are most applicable to the branding of hardware and software used in 3D bioprinting and nanotechnology.

19.3.4 TRADE SECRETS

Trade secrets¹³ are confidential business information that provide a competitive advantage and have some commercial value.¹⁴ A trade secret can take the form of a formula, program, device, technique, manufacturing process, or any other nonpublic information that provides a competitive advantage. Trade secret protection is most effective when a successful independent discovery or reverse engineering is very difficult to accomplish without having the trade secret information. Because such difficulty results in a low likelihood of successful copycats, the trade secret owner can keep their innovations a secret, and forego the patenting process which requires disclosure to the public. Some of the most famous trade secrets are secret formulas and recipes, like the Coca Cola formula and the Kentucky Fried Chicken recipe.¹⁵ These recipes are locked away, under heavy guard, and only known to a very select few. Indeed, many have tried to decipher the recipes themselves, but no one has succeeded to the point where Coca Cola or KFC loses distinction. Therefore they maintain a competitive advantage by keeping their recipes secret.



No formal registration or application process for trade secrets exists, and protection attaches immediately. Trade secret protection can last indefinitely, so long as the owner keeps the business information a secret.¹⁶ To protect a trade secret, the owner must take appropriate steps to maintain its secrecy and avoid public disclosure or theft. The sufficiency of these steps depends on a number of factors, such as the likelihood the secret would be stolen or the amount of interest from competitors in the trade secret. In the Coca Cola example, many beverage companies would pay millions to obtain the original formula, and many protection measures are needed to protect the formula from theft or public disclosure. Unlike patent protection, independent discovery by others is allowed. If another person successfully reverses engineers or independently creates the trade secret,

¹³Image from <http://www.corporatecomplianceinsights.com/want-a-cost-effective-way-to-bolster-compliance-with-export-controls-look-to-your-trade-secret-procedures/>.

¹⁴World Intellectual Property Organization. *What is a Trade Secret?* http://www.wipo.int/sme/en/ip_business/trade_secrets/trade_secrets.htm.

¹⁵Kane, C. (2012). *7 Sought-After Trade Secrets*, Published August 22, 2012, <http://www.cnn.com/id/48755451>.

¹⁶World Intellectual Property Organization, *How are Trade Secrets Protected??* http://www.wipo.int/sme/en/ip_business/trade_secrets/protection.htm.

then trade secret protection ends, as the trade secret is deemed “public information.” However, if someone attempts to *steal* the trade secret information, the trade secret owner can stop the thief from using the information and recover for losses from the trade secret theft, so long as the trade secret owner can prove the secret was stolen despite appropriate measures to maintain secrecy.

19.4 WHERE DOES INTELLECTUAL PROPERTY LAW ORIGINATE?

Modern IP laws originate from the US Constitution and acts of Congress. Multiple clauses in the Constitution direct the US government to make laws that protect the rights of individuals who contribute to the greater good through innovations and creative works. Article 1, Section 8, Clause 8 of the Constitution provides Congress with the broad power “to promote the progress of science and useful arts, by securing for limited times to authors and inventors the exclusive right to their respective writings and discoveries.” This clause forms the basis of the US Patent and Copyright laws. Article 1, Section 8, Clause 3 of the Constitution provides the basis for federal trademark law, by providing Congress with the broad power “to regulate commerce with foreign nations, and among the several states, and with the Indian tribes.” State laws also protect trademarks. Trade secret laws are creatures of state law, and most states have adopted the Uniform Trade Secrets Act.



19.5 WHAT ASPECTS OF 3D BIOPRINTING AND NANOTECHNOLOGY ARE PROTECTABLE?

3D bioprinting and nanotechnology are complex technologies that combine specialized computerized machinery, specialized materials, and intricate processes of additive manufacturing and nano-scale manufacturing. Many aspects of 3D bioprinting and nanotechnology for tissue engineering may be protected by IP, including improvements on technologies that are already protected and innovative new technologies that do not fit squarely within current IP laws. Indeed, IP laws may apply to nearly all aspects of 3D bioprinting and nanotechnology, including the hardware used to print an item or handle nanoparticles, the software involved in the design of tissue structures and the operation of the hardware to create the tissue, and specialized materials used to form or process the nanostructures or created tissue. 3D bioprinters combine cutting-edge machinery, electronic circuitry, software, materials, and processes in each creation of tissue, and the resulting tissue and organs may be protectable as well.

Historically, implanted devices, surgical treatments and procedures, and pharmaceutical drugs have been protected by utility patents, design patents, trademarks, copyrights, and trade secrets.

Implanted devices fall squarely within the “machine” category of utility patent laws, and possibly design patents for the ornamental design of the implanted devices. Surgical treatments and procedures, although not protectable in some countries, fall within the “process” category of the US utility patent laws. Pharmaceutical drugs have been protected by utility patents for “compositions of matter,” where the patents are granted for the man-made chemical structures of the drugs. Other medical inventions are also afforded IP protection although they do not fall squarely within the original IP law definitions. For example, although subject to much controversy, nonhuman organisms, tissue, and even human genes have been patented, subject to limitations discussed below.

The most prominent forms of IP involved in 3D bioprinting and nanotechnology are patent and trade secret protection, discussed in detail below. Copyrights also protect the software executed by 3D bioprinting and nanotechnology machinery. To better understand what new aspects of 3D bioprinting and nanotechnology can be protected, this chapter also addresses the aspects of tissue engineering and bioengineering currently protectable under the US IP laws.

Section 101 of the US patent laws broadly defines a patentable invention as a “new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof.”¹⁷ For a long time after The Patent Act of 1790 established the US patent system, patents were granted for new and improved machines and methods for creating or physically transforming objects. The advent of computers brought waves of patent applications for computers and for computer software. Now, software such as medical diagnostic programs, device control programs, and computer-aided design programs are patentable for the functions that processors perform while executing the software and even for the physical memory or media on which the software is stored. 3D bioprinting and nanotechnology combine many known technologies with groundbreaking and unknown technologies and open new frontiers in IP. The main categories of patentable aspects of these technologies are hardware, software, processes, and materials.

19.5.1 HARDWARE

The devices involved in creating 3D bioprinted tissue or nanostructures fit squarely within the “machine” category of patentable subject matter. Of course, 3D printers are machines whose functionality can be protected by utility patents. A single piece of hardware can be protected by multiple patents covering different parts or part combinations of the device. Within a single 3D bioprinter, there may be numerous systems working to move print heads, process raw materials such as printable cells and binding agents, lay the raw materials, and perform additional processing to preserve the printed tissue. Whereas basic 3D printers heat plastic filament and lay melted plastic in patterned layers, 3D *bioprinters* must store, handle, and construct biological materials, a much more demanding manufacturing environment that requires the hardware needed to create live tissue, a much more difficult task than simply layering melted plastic.

Indeed, 3D bioprinters may handle many types of biological and nonbiological materials and may require specialized reservoirs to store living cells and biological binding agents and specialized hardware to treat printed tissue with nanomaterials or other materials to produce biocompatible tissue and organs. The nanomaterial handling hardware may be incorporated into 3D bioprinters to coat printed tissues with nanoparticles. As one would expect, the hardware involved in creating live

¹⁷*Inventions Patentable*, 35 U.S.C. Section 101.

tissue is very complex, and the innovators and investors paving the future require assurance that their investments in successful innovations will not be usurped by copycats.

Another important piece of hardware used in 3D printing is the 3D scanner. Scanners simplify and expedite the process of replicating objects, including living tissue. 3D scanners have existed for years as patentable machines,¹⁸ but as tissue engineering improves with the use of 3D bioprinting and nanotechnology, scanning technology will also improve. 3D scanners can scan a piece of living tissue, determine its cellular structure and composition, and generate instructions for a 3D bioprinter to replicate the tissue. All of these functions, as well as the hardware used to perform the functions, can be protected by utility patents, design patents, and trade secrets.

19.5.2 SOFTWARE

3D bioprinters and nanomaterial handling devices are often highly automated computerized machines. The complex process for printing and processing living tissue involves many factors that must be continuously monitored and controlled, including temperature, acidity, wetness, oxygenation, and nutrition for the printed cells. Fabricating nanostructures involves precise material handling and deposition. Even relatively basic 3D printers that extrude plastics to build biodegradable scaffolds for shaping cell growth must heat plastics to specific temperatures, navigate one or more print heads within a three-dimensional environment, and extrude a specific amount of plastic, all with high precision. These complex, repetitive steps are performed automatically by the programmed 3D bioprinter or nanostructure fabricator, and the software controlling each of these devices may be protectable by both patents and copyrights.

To revisit the distinctions between copyrights and patents for software, copyright protection prevents anyone from copying source code from the author's program or using the same source code in different programs without the author's permission. Copyrights do not prevent competitors from developing functionally equivalent software using different codes. Software patents can protect the functionality and protect against competitors developing software having the patented functions. For example, software patents can protect the functional aspects of a 3D printer or nanostructure fabricator, such as the steps performed by the device processors executing the software, and the settings used to produce a 3D bioprinted product or nanostructure. Patenting the device software allows the innovators to prevent others from copying the software and making insignificant changes to the code that may evade copyright protection.

Software for creating digital "blueprints" for 3D printed products and nanostructures, and the digital blueprints themselves, may be also protected by patents and copyrights. These digital blueprints can be created automatically using 3D scanners (and 3D scanner software) or manually using computer-aided design software developed especially for tissue engineering and nanoscale fabrication. Again, copyrights would protect the specific source code underlying the software, whereas patents can protect the functionality of the software and a broader description of the 3D printer instructions for creating the tissue, such as dimensions, cellular arrangement, and other operational parameters.

¹⁸See, for example, *Optical 3D digitizer, system and method for digitizing an object*, US Patent No. 6,294,095, issued December 10, 2000.

19.5.3 METHODS/PROCESSES

Method patents can protect, at a high level, the entire process of creating a nanostructure or 3D bioprinted object, including computerized steps for controlling hardware, the physical actions performed by the hardware, and even manual steps performed before or after the computerized fabrication. Therefore method patents can provide broad IP coverage of the steps involved to prepare the raw materials, construct the tissue or nanostructure, and perform postprocessing steps, such as treating the printed tissue with nanomaterials to enhance biocompatibility or drug delivery capabilities. Unlike patents and copyrights covering software, method patents can cover computerized steps and also manual steps involved in the creation of printed tissue. Patents covering the manufacturing process are often appropriately referred to as “methods of manufacture.” These patents can cover the entire manufacturing process or just a portion of the process. An example of a method patent covering a portion of a bioprinting process could cover an improved method for storing and transporting living cells before 3D bioprinting occurs. Another patent could cover a method of laying and binding living cells. Yet another patent could cover a method of treating the printed tissue with nanomaterials. Indeed, a single manufacturing process may be protected by several patents covering different portions.

Tissue engineering and nanofabrication methods are also ideal for trade secret protection, especially methods involving highly specialized (and also secret) hardware, or manual steps known only to the innovator. Because of the complex nature of engineered tissue and organs, reverse engineering a printed organ to determine how the cell structures were formed and to determine how the tissue was processed to increase biocompatibility may be difficult to accomplish. Reverse engineering nanofabrication methods is also extremely difficult due to the miniature and complex nature of nanostructure products. Accordingly, innovators who do not want to divulge any of their processes to the public in a patent can hold them as trade secrets and fully capitalize on their inventions until competitors reverse engineer their method or come up with the next best method.

19.5.4 MATERIALS

3D bioprinted and nanofabricated products use some entirely new materials. For example, printed tissue requires specialized materials to form the tissue structures and process the tissue for increased biocompatibility or drug delivery properties. The man-made and artificially modified materials used for 3D bioprinting and nanotechnology are patentable because they are “compositions of matter.” Tissue printed from a 3D bioprinter is usually formed using “bioink,” a medium of living cells suspended in a liquid that protects the cells and keeps them alive, and helps the cells adhere to the tissue structure during printing. Some forms of bioink use natural materials or natural cells such as stem cells. While the natural materials and cells themselves may not be patentable, artificial compounds that happen to include natural materials, as well as artificial modifications of the natural materials, can be patented. For example, some types of bioink use natural gelatins as the printing medium. Natural gelatin is semisolid in its natural state, but the gelatin used in bioink is modified to remain in liquid form during storage and printing and only hardens during printing and postprocessing of the printed tissue.¹⁹ The modified gelatin, which has different chemical

¹⁹Maxey, K. (2013). *Gelatin Bio-Ink Could Lead to 3D Printed Organs*, Published November 4, 2013, <http://www.engineering.com/3DPrinting/3DPrintingArticles/ArticleID/6585/Gelatin-Bio-Ink-Could-Lead-to-3D-Printed-Organs.aspx>.

properties, is patentable as a composition of matter. Like the modified gelatin, bioink that incorporates natural living cells is patentable as a composition of the matter when the living cells are mixed or treated with artificial compounds to form combinations that are not found in nature. For example, the living cells in the bioink may be treated or processed to enhance biocompatibility, adhesion to other cells, or prolong the shelf life of the bioink or printed tissue.

Patents on the bioink, nanomaterials, and other materials used in 3D bioprinting may also tie into patents on the reservoirs and hardware that hold the bioink and keep the living cells alive, the mechanisms that transport the cells, and the specialized print heads that dispense bioink. Specialized patentable materials may also be used in these hardware components, to keep the cells alive and intact while printing tissue. Materials used to construct the 3D bioprinting hardware may be patentable, even if the bioink is all-natural and thus unpatentable.

Some types of 3D bioprinters do not print cells to create engineered tissue and organs. Instead, the 3D bioprinter creates a biodegradable structure, called a “scaffold,” from biodegradable materials, which are then covered with cultured living cells to grow into the tissue or organ structure. The living cells may not be patentable if they are simply cultured natural cells, but man-made or artificially modified scaffold materials may be patentable. Furthermore, materials and processes that improve scaffold materials to enhance biodegradability and increase adhesion to the living cells may be patentable processes and compositions of matter.

19.6 INTELLECTUAL PROPERTY PROTECTION LIMITATIONS FOR ENGINEERED TISSUE

The previous section addresses aspects of 3D bioprinting and nanotechnology that are protectable by patents, copyrights, and trade secrets. While there is a broad range of IP protection available, modern laws and court decisions have carved out some exceptions to IP protection for public policy reasons. For example, the public would not benefit from granting monopolies over things that can be found in nature or scientific principles such as $E = MC^2$. This section explores in more detail the limitations on aspects of tissue engineering with 3D bioprinting and nanotechnology that are subject to IP protection and the exceptions to protection.

Just as the bulk of IP protection for 3D bioprinting and nanotechnology technologies is patent protection, the majority of exceptions to IP protection in these industries fall under exceptions to patentability. Section 101 of the US patent laws broadly defines what can be patented, but the US Patent Office and the courts have carved out numerous exceptions and limitations.²⁰

The most important exception to patentability is the human body. Courts have always prohibited patents on the human body and human organisms as violations of the Constitution, because granting property rights in the human body is akin to a form of slavery.²¹ The recently enacted America Invents Act codified the ban on human patents, stating “[n]otwithstanding any other provision of law, no patent may issue on a claim directed to or

²⁰United States Patent and Trademark Office, *Patents*, <http://www.uspto.gov/inventors/patents.jsp>.

²¹Torrance, A. (2013). *Nothing under the sun that is made of man*, Published February 7, 2013, <http://www.scotusblog.com/2013/02/nothing-under-the-sun-that-is-made-of-man/>.

encompassing a human organism.”²² This exception falls under the umbrella of a broader limitation on IP: that one cannot hold IP rights on things found in nature. However, nonhuman organisms, animal tissues, and even human genes have been patented for years and are considered patentable subject matter so long as they are man-made or man-modified into something that does not occur in nature.²³

Engineered tissue and organs created with 3D bioprinters and nanotechnology will probably remain patentable to the extent that the tissue is considered “manmade.” Manufactured tissue, such as 3D bioprinted tissue, is artificially created by assembling cells and not by natural growth. Tissue grown in Petri dishes may be deemed unpatentable because it grew by natural processes, albeit with the assistance of man. But a machine undoubtedly constructs printed tissue with the use of artificial materials mixed with living cells. The resulting printed tissue is considered to be “man-made” for the purpose of IP protection. Ethical considerations related to IP rights on tissue and organs are discussed below.

An argument can be made that tissue grown over 3D printed scaffolding is “natural” and therefore that the grown tissue cannot be protected, but at the very least variations of the process of creating that tissue—printing the scaffolding, applying the cells, and growing the culture—is considered a patentable process.²⁴ Additionally, the legal restrictions on patenting human organisms do not categorically exclude human tissue, organs, and other human organism components from being protected by patents.²⁵

More questions about the patentability of engineered tissue arise when 3D printed tissue and organs are indistinguishable from natural organs and tissues. Should man-made tissue and organs be patentable when they are identical in form and function to natural tissue and organs? Of course, any patents on tissue and organs could only cover the man-made versions, but discerning between infringing products and naturally grown tissue could be extremely difficult when 3D printing and nanotechnology are able to produce exact replicas of natural tissue and organs. One could argue that these products of 3D printers are essentially the same as a naturally occurring organ or tissue and should be regarded as “naturally occurring” for the purpose of patentability. Others could argue that the tissue and organs, although replicas of nature, are still “articles of manufacture” and should be patentable.

Science has yet to progress to the level where 3D bioprinted tissue is completely indistinguishable from natural tissue, but at some point, this IP dilemma will emerge. One possible solution for

²²Limitation on issuance of patents, America Invents Act (2011), Section 33.

²³According to *Chisum on Patents* (2014, appendix 24), “To the extent that the claimed subject matter is directed to a nonhuman “nonnaturally occurring manufacture or composition of matter—a product of human ingenuity” (*Diamond v. Chakrabarty*), such claims will not be rejected under 35 U.S.C. Section 101 as being directed to nonstatutory subject matter.”

²⁴*Chisum on Patents* (2014, Section 1.02 [7] Product of Nature-Biological Subject Matter, discussing *SC Computer Prods. v. Foxconn Int’l*, 355F.3d 1353, 1359 (Fed. Cir. 2004)), “An invention synthesized for the first time in a laboratory is eligible for patent protection under Section 101. Processes for producing this synthetic product in the laboratory and/or for using this synthetic product may also be eligible for patent protection under Section 101. However, a natural reproduction process, whether sexual, asexual, part of a chain reaction, or a process of decay, is ineligible for patent protection under Section 101... An item reproduced by such a natural process, whether an inorganic structure or a life form, must ipso facto be ineligible for patent protection under Section 101.”

²⁵Kapmar, D. (2013). *A look at the patentability of 3-D printed human organs*, Published May 28, 2013, <http://www.law360.com/articles/439549/a-look-at-the-patentability-of-3-d-printed-human-organs>.

this potential issue is marking or branding the fabricated tissue and organs to distinguish patented man-made products from naturally grown tissue and organs, such as a DNA marker.²⁶ By purposefully distinguishing man-made tissue from natural tissue, innovators may continue to protect the IP of their inventions without challenging current patent laws.

19.7 ETHICAL CONSIDERATIONS OF ENGINEERED TISSUE INTELLECTUAL PROPERTY

Despite the legality of IP protection for engineered tissue, moral implications of 3D bioprinting may lead to spirited debate over the patentability of tissue, organs, and other medical products.²⁷ Patents grant the right to exclude others from making, using, or selling the protected product or method. Some view this exclusivity as a monopoly on the protected product or method, a monopoly that is undesirable when public health and access to treatment may be adversely affected. While innovations in 3D bioprinting and nanotechnology can improve the speed and quality of tissue and organ production, should society grant the innovators the right to exclude others from creating human body parts?²⁸

The FDA, which evaluates and approves medical devices, drugs, and treatments, may impose strict guidelines for approving 3D bioprinted tissue and organs or ban them altogether when there is uncertainty regarding the consistency, quality, or safety of 3D bioprinted tissue made in hospitals and doctors' offices. Perhaps IP can help reduce this uncertainty, by granting patents to the innovators with particular tissue designs, manufacturing methods, and organ products. The patent owners' right to exclude others would mean that the FDA could focus its efforts on ensuring the patented product or method is safe and yields consistent results, even when the tissue is printed in individual medical facilities. Indeed, the freedom to print tissue and organs in individual offices and hospitals using unregulated and untested product designs and methods may result in inconsistent healthcare with the potential for harmful results and/or heavy regulation.

As discussed above, limited "monopolies" created by IP are necessary in the medical and biological fields to allow inventors and investors to recoup upfront R&D expenses and to help fund future R&D, thus furthering the progress of science. This model has driven the pharmaceutical industry for decades, granting drug makers a temporary monopoly on a particular drug before allowing generic brands to start selling the same drug.²⁹ The patent owner, in this case a

²⁶See Homick, J. (2014). *How to Tell What's Real and What's Fake in a 3D Printed World*. 3D Printing Industry, February 5, 2014, http://3dprintingindustry.com/2014/02/05/tell-whats-real-whats-fake-3d-printed-world/?utm_source=3D+Printing+Industry+Update&utm_medium=email&utm_campaign=38c9fa9fa0-RSS_EMAIL_CAMPAIGN&utm_term=0_695d5c73dc-38c9fa9fa0-60484669.

²⁷Gartner, Inc. (2013). *Gartner Reveals Top Predictions for IT Organizations and Users for 2014 and Beyond*, Published October 8, 2013, <http://www.gartner.com/newsroom/id/2603215>.

²⁸Gartner, Inc. (2014). *Gartner Says Uses of 3D Printing Will Ignite Major Debate on Ethics and Regulation*, Published January 29, 2014, <http://www.gartner.com/newsroom/id/2658315>.

²⁹Schacht, W.H., & Thomas, J.R. (2005). *Patent Law and its Application to the Pharmaceutical Industry: An Examination of the Drug Price Competition and Patent Term Restoration Act of 1984*, CRS Report for Congress, Published January 10, 2005, Available at <http://www.law.umaryland.edu/marshall/crsreports/crsdocuments/rl3075601102005.pdf>.

pharmaceutical company, bears the burden of accountability and ongoing testing by the FDA and health organizations and enjoys any resulting profits from sales of the patented drug. In some cases, this allows the drugmaker to set the price and to control the drug supply, which could have the effect of limiting accessibility to affordable treatment. But without this limited monopoly, drug quality could be inconsistent and the healthcare industry could suffer from too many versions of the same drug, and accountability could be spread over many pharmaceutical companies.

Granting an innovator the right to exclude others from making or using the invention allows the public to hold the easily identified patent owner accountable if something goes wrong. The ability to hold an identifiable entity accountable for harm and negligence is very important when public health is at stake, as in the medical and healthcare industries. An IP owner is easier to identify as the responsible party when something goes wrong, whereas, in contrast, everyone points fingers when companies are allowed to copy ideas from one another and something goes wrong.

There are many arguments for and against granting IP rights for 3D printed tissue and organs. IP generally fosters innovation and can serve societal interests by yielding consistent, quality products with high accountability. Some view IP protection as a burden on society, because IP protection can increase product costs and can limit product availability by granting exclusive rights to the IP holder. Although one innovator may hold a limited monopoly over a certain tissue structure or method, competitors are free to improve upon the patented technology. Competitors can also benefit from the patent's disclosure in developing their own products that may be eligible for IP protection. As tissue and organs created with 3D bioprinting and nanotechnology become more mainstream and refined, these ethical debates will surely become more frequent and prominent, and society will decide whether IP rights are suited for engineered tissue.

19.8 INTELLECTUAL PROPERTY INFRINGEMENT

IP laws are designed to prevent copycats and to advance the progress of science, but these laws are not perfect. Indeed, IP laws alone are not 100% efficient in deterring copycats from trying to make quick profits without any contribution to society. To protect their investments and innovations, IP owners often need to enforce their rights and prosecute infringers.

Infringement involves the unauthorized use or sale of products and processes protected by IP. Different types of IP have different standards and requirements for proving infringement, but for any type of IP, the property owners have the responsibility of enforcing their rights. The US Patent and Trademark Office and the US Copyright Office do not monitor and police infringing activity.³⁰

19.8.1 TRADEMARK INFRINGEMENT

Trademark infringement occurs when someone uses a mark, such as a word, logo, slogan, or other identifying mark as his or her own, without permission from the owner of the mark. Infringement

³⁰United States Patent and Trademark Office. *General Information Concerning Patents: Infringement of Patents*, Updated November 2011, http://www.uspto.gov/patents/resources/general_info_concerning_patents.jsp#heading-28; United States Copyright Office, *Services of the Copyright Office*, Revised March 1, 2012, <http://www.copyright.gov/help/faq/faq-services.html#advice>.

causes confusion among consumers, who are not able to distinguish between products and services made and sold by the owner of the trademark and products and services made and sold by the trademark infringer. The end result may be lost profits and a tarnished reputation for the trademark owner. In tissue engineering, the end product—the tissue and organs themselves—may not be subject to trademark infringement, unless the tissue or organs are branded. This may or may not happen. At this time, trademark protection is more appropriate for the hardware used to create 3D printed tissue and organs.

19.8.2 TRADE SECRET MISAPPROPRIATION

Trade secret infringement (called “misappropriation”) occurs when a trade secret, such as a secret method of treating tissue with nanomaterials, is stolen from the trade secret owner and used for unfair competition. To allege trade secret misappropriation, the trade secret owner must first prove that a trade secret existed by showing that (1) it was not generally known to the public, (2) it conferred a competitive advantage to the owner, and (3) reasonable steps were taken to maintain secrecy.³¹ However, if the accused misappropriator can show independent development of the same method without using the trade secret, such independent discovery is a complete defense, and there is no misappropriation.

Applying these principles to tissue engineering, an innovator may invent methods for creating or treating tissue to exhibit certain desirable properties, such as high biocompatibility or extraordinary drug delivery capabilities. The innovator could hold a trade secret if the method is not publicly known or apparent from simply observing the tissue. The innovator would have the ability to sue thieves who learned of the method by stealing the innovator’s lab notebook, hacking into the innovator’s computer where the method was documented, or leaving the innovator’s employ with knowledge of the secret process and setting up a competing company. However, the innovator would not be able to sue another innovator who independently came up with the same method. The independent discovery defense is one of the caveats to trade secret protection. As discussed below, patent protection is immune to the independent discovery defense, albeit at the cost of disclosing the secret method to the public.

19.8.3 COPYRIGHT INFRINGEMENT

To prove copyright infringement, the copyright owner must show that its copyrighted work was reproduced, distributed, performed, publicly displayed, or made into a derivative work without the copyright owner’s permission.³² In 3D bioprinting and nanotechnology, copyright laws mainly protect the software used in computerized equipment and potentially protect the digital blueprint files that the 3D bioprinter uses to create tissue and organs.³³ Thus detecting copyright infringement in

³¹Cornell University Law School. *Trade Secret*. Legal Information Institute, http://www.law.cornell.edu/wex/trade_secret.

³²United States Copyright Office. *Definitions*, <http://www.copyright.gov/help/faq/faq-definitions.html>.

³³Current law probably does not favor copyright protection of the digital blueprint of functional products, such as tissue and organs, but this could change.

3D bioprinting involves comparing the copyright owner's software to the potential infringer's software.

While detecting software copyright infringement can be as easy as comparing source code, enforcing copyrights against 3D printing software may be a daunting task. 3D printing lends itself to decentralized manufacturing systems in which individuals use their own printer to create products on-demand and on the spot. Because the products themselves may not enter a distribution chain, such as from manufacturer, to distributor, to customer, 3D printed product sales may not be regulatable like products that you would buy in stores. Instead, 3D bioprinters in hospitals or doctors' offices may be used in two scenarios: (1) the doctor designs his/her own tissue or organ using specialized software, and 3D prints the tissue/organ, or (2) the doctor downloads a digital blueprint for a certain organ or tissue, and 3D prints the tissue/organ, with or without changes.

In the first scenario, the software used to create the tissue/organ design and the software used to control the printer to create the tissue/organ are subject to copyright protection. Copyright infringement may be relatively easy to enforce in this scenario because the software would be distributed from a known source.

In the second scenario, the 3D printer software and possibly the downloaded digital blueprint are subject to copyright protection. As discussed above, the 3D printer software is less prone to copyright infringement when obtained from a known source. However, copyrights on digital blueprints may be difficult to enforce when the files are distributed over the Internet, such as between medical professionals. If the digital blueprints are not copyrightable, copyright may not be an option for extracting profit from digital tissue and organ blueprints.

Like the problems faced with MP3 file sharing in the 1990s and 2000s, digital blueprint file sharing can include unregulated and unlicensed distribution of IP. The innovators who design successful 3D printable tissue structures and organs stand to lose profits and recoupment of R&D expenses when their digital blueprints are distributed freely and used without collecting any royalties. Like many demoralized musical artists, unchecked file sharing of digital blueprints could disincentivize the innovators in tissue engineering, especially if they are not copyrightable. But unlike MP3 file sharing issues, the end users of the 3D bioprinting digital blueprints are sophisticated hospitals, laboratories, and doctors who would stand to lose a lot from copyright infringement lawsuits. Therefore the sophistication of the medical and healthcare industries, coupled with ever-emerging protection mechanisms in digital files (known as digital rights management, or DRM), may keep unauthorized copying at a manageable level that does not significantly stifle innovation.

19.8.4 PATENT INFRINGEMENT

Patent infringement is probably the most important type of IP enforcement in the United States and throughout the world. Proving patent infringement requires showing that an unlicensed, unauthorized entity is making or selling a product or service covered by the patent. The patent owner does not need to prove that the alleged infringer knew about the patent, but proof of prior knowledge can elevate the patent infringement claim to *willful* infringement, a more serious allegation with harsher consequences. Willful infringers are true copycats who simply steal a patented idea and pass it off as their own. Under the US patent law, even others who independently create the patented device (and are therefore not willful infringers) are still liable for patent infringement, and

their independent efforts alone cannot defeat a claim of patent infringement.³⁴ Patent protection provides the ability for the patent owner to exclude *anyone* from making or selling a patented invention, as one of the incentives for convincing inventors to disclose their inventions to the public when obtaining the patent.

Patent infringement in 3D bioprinting and nanotechnology may be extremely difficult to detect. Usually, patent owners watch open markets to identify infringing products and trace the infringing product back to the patent infringer. For example, a 3D bioprinter patent owner may look for competing machines with the configuration of the same parts as the configuration in the patent owner's patent. Similarly, a method patent owner may check products in the open market to see whether a competitor appears to be using the patented method without authorization, and a design patent owner may enforce patent rights against infringers making or selling products that look like the patented design. 3D bioprinting, however, and more particularly 3D bioprinted tissue, present additional obstacles to detecting patent infringement.

Patents, like any IP, are only useful when the patent owner is able to enforce IP rights and exclude others from making and selling the patented item. Enforcing IP rights requires identification of the infringing products and the infringers, usually after the infringing product enters the open market. However, 3D bioprinted tissue, like any 3D printed item, may be created on-demand and on the spot. A hospital or doctor's office could print the tissue at the time it is needed, such as immediately before surgery. The printed tissue or organ could be implanted into a patient at the same location, and thus printed tissue and organs may never enter the open market. Even once implanted, patented tissue and organs, like all patented implants, are still subject to IP laws.³⁵ For a while, 3D bioprinted tissue and organs probably will be manufactured in centralized locations, but once bioink and tissue digital blueprints are readily available, most 3D bioprinters probably will be located in individual labs, hospitals, and doctors' offices, and tissue and organs will be printed on-demand. For patent owners, this may present an enforcement nightmare, trying to identify patent infringers of 3D printed tissue and organ patents. The printed tissue and organs, once implanted in patients, will be impossible to detect and analyze for possible infringement. The person sitting next to you could have implanted, infringing 3D bioprinted tissue or organs, but it would be extremely difficult, if not impossible, to detect. This obstacle to detecting infringement also applies to conventional implanted devices, such as pacemakers, but such devices may continue to be manufactured in centralized locations and distributed in the open market, increasing their visibility to the public and to patent owners, at least in the short term. Therefore patents on engineered tissue and organs made with 3D printers and nanotechnology may be very valuable IP assets, but they may also be very difficult to enforce against infringers.

Although most 3D bioprinted tissue and organs may not be distributed in open markets, merchants will distribute raw materials, such as bioink and nanomaterials, 3D bioprinter and nanotechnology hardware, and control software. Patent infringement of such 3D bioprinting and nanotechnology technology may be easier to detect, and thus innovators in raw materials, hardware, and control software used in 3D bioprinting and nanotechnology may be able to track down

³⁴Cornell University Law School. *Trade Secret*, Legal Information Institute, <http://www.law.cornell.edu/wex/patent>.

³⁵*Monsanto Co. v. McFarling*, 488F.3d 973, 2007 US App. LEXIS 12099 (Fed. Cir. 2007), discussed Section 5640, Section 6213 ("The principles of patent law do not cease to apply when patentable inventions are incorporated within living things, either genetically or mechanically.").

infringers easier and enforce their patents more effectively. However, digital blueprints for tissue and organs, even if patentable, may be shared peer-to-peer, or tissue and organs may be made and sold from such blueprints on the black market, without any way for the patent owner to detect infringing uses of the patented products.³⁶

19.9 Conclusion

IP is an important part of the evolving technologies of 3D bioprinting and nanotechnology. As tissue engineering and nanofabrication methods continue to improve, including hardware, software, materials, and processes, innovators will look to IP to protect their innovations from exploitation by others and to capitalize on their contributions to society. By enforcing their IP rights, innovators will seek to recover their financial investments to fund further research, and continue advancing the progress of science. Although there is some uncertainty as to the scope and ethical considerations of IP protection for engineered tissue, many aspects of 3D bioprinting and nanotechnology are protectable under current laws and will likely remain protectable for the foreseeable future.

QUESTIONS

1. What are the four main types of intellectual property in the United States?
2. How are trade secrets protected, and what factors are involved in protection?
3. What types of 3D bioprinted products would not be eligible for patent protection?
4. What benefits does society receive from the patent system?
5. What aspects of 3D bioprinting and nanofabrication can be protected by copyright?

ANSWERS TO QUESTIONS

1. Patents, Copyrights, Trademarks, and Trade Secrets.
2. The owner must take appropriate steps to maintain its secrecy and avoid public disclosure or theft. Factors in the sufficiency of these steps can include the likelihood the secret would be stolen or the amount of interest from competitors in the trade secret.
3. Printed tissue and organs that are indistinguishable from natural tissue.
4. Patent owners can be readily identified, and the public can hold patent owners accountable if something goes wrong with the patented technology. The patent disclosure can also assist others in developing products that improve upon the patented technology.
5. The software that configures and runs 3D bioprinters or nanofabrication devices and, potentially, the digital “blueprints” for printed and fabricated products.

³⁶See Lipson, H., & Kurman, M. (2013). *Fabricated: The New World of 3D Printing*; John Wiley & Sons, Inc., pp. 1–4.

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